IMMUNOGENETIC ADAPTATION TO AN EMERGENT AMPHIBIAN DISEASE

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IMMUNOGENETIC ADAPTATION TO AN EMERGENT AMPHIBIAN DISEASE

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The disease chytridiomycosis, caused by the fungal pathogen
*Batrachochytrium dendrobatidis* (*Bd*), emerged in the 1970s and has caused the
delay and extinction of hundreds of amphibian species worldwide. Several
pathogen and environmental factors have been identified that play critical roles in
determining *Bd* disease dynamics. In comparison, host factors have been infrequently
characterized and remain poorly understood. Here, I explore genetic responses to *Bd*
in the lowland leopard frog (*Lithobates yavapaiensis*) to evaluate the hypothesis that
host genetic factors contribute to *Bd* susceptibility across natural populations. I
characterize disease prevalence, environmental variables, and measures of genetic
variability in eleven natural populations to reveal spatial and temporal *Bd* dynamics. I
also perform experimental *Bd* infections in lab-reared frogs collected from five natural
populations. For both experimentally reared frogs and natural populations, I
characterize allelic variation at an expressed Major Histocompatibility Complex (MHC)
class IIB locus that encodes peptides that initiate acquired immunity. I find that
infections are minimal in summer but abundant in winter, some populations are *Bd*
infected without developing chytridiomycosis, and other populations are *Bd* infected
and experience fatal bouts of chytridiomycosis. I identify an outlier locus that shows
associations to *Bd* susceptibility, and find the best models predicting *Bd* dynamics
include both genetic diversity and environmental variables. I show that MHC alleles
associate with surviving *Bd* infection in both lab-infected frogs and naturally sampled
individuals. Individuals bearing MHC allele Q show significantly reduced risks of death, and I detect positive selection along the evolutionary lineage leading to allele Q. Further, in one Bd resistant population, I detect a significant signal of directional selection for allele Q. For lab-infected frogs only, MHC heterozygotes also have a significantly reduced risk of death. In summary, I find that population genetic and immunogenetic variation contributes to Bd susceptibility after controlling for environmental variation, demonstrating that host genetics significantly affect chytridiomycosis outcomes and may be a powerful tool for conserving global amphibian biodiversity.
BIOGRAPHICAL SKETCH

Anna Evangeline Savage was born on the fourth of July, 1982, in the town of Barrington, New Hampshire, USA. She grew up in Northwood, New Hampshire, and was homeschooled through elementary school, graduated from Coe-Brown Northwood Academy in 2000, and graduated *summa cum laude* from Amherst College in 2004 with a B.A. in Biology.
To my father Jack Savage

He knows what he did.
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Unexplained amphibian declines began catching the attention of biologists as early as the 1970s. By 1989, population declines without obvious environmental or anthropogenic causes had become so abundant, conspicuous, and globally distributed that scientists convened for the first World Congress of Herpetology to discuss the mysterious frog and salamander disappearances (Barinaga 1990). Nine years of concerted research effort passed before the culprit was definitively identified to be the disease chytridiomycosis (Berger et al. 1998) caused by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore et al. 1999). The pathology and progression of chytridiomycosis is now well understood, and biologists have identified numerous factors influencing *Bd* disease dynamics. However, epidemiological knowledge of *Bd* has yet to temper the effects of this emerging pathogenic disease. At present, a minimum of 350 amphibian species have been infected with *Bd* (Fisher et al. 2009), more than 200 species have declined or gone extinct (Skerratt et al. 2007), and *Bd* continues to be detected in new regions and species worldwide (Savage et al. 2011; Bell et al. 2011).

Chytridiomycosis is a single-pathogen, multi-host disease that occurs on every continent with amphibians (Europe [Bosch et al. 2001], Africa [Hopkins & Channing 2003], Australasia [Berger et al. 1998; Waldman et al. 2001], Central [Lips 1999; Lips et al. 2003], South [Bonaccorso et al. 2003; Ron et al. 2003], and North America [Bradley et al. 2002; Green et al. 2003]). This complex combination of interacting factors presents a challenge for isolating common causes and consequences of disease severity and pathogenicity. The disease triangle (Scholthof 2007) is a useful model for exploring interactions between the three components that contribute to all diseases: the host, the pathogen, and the environment. Studies investigating one corner of the triangle while...
controlling for the other two can identify important elements that may be universal for chytridiomycosis. Conversely, studies exploring the interactions between all three disease components can provide a more realistic picture of the critical epidemiological factors for a specific combination of host, pathogen and environment. A summary of current knowledge for each corner of the chytridiomycosis disease triangle is presented below, followed by a discussion of the most critical areas needing more research.

**Pathogen Contributions**

The origin of *Bd* and causes for its emergence as an amphibian pathogen are still debated. Spatial-temporal patterns of outbreaks suggest that *Bd* is a novel introduced pathogen that is spreading regionally (Morgan *et al.* 2007; Lips *et al.* 2008) while other analyses indicate that climate-linked changes in pathogenicity may have facilitated localized outbreaks of endemic *Bd* strains in amphibian populations (Pounds *et al.* 2006). Consistent with both of these hypotheses, frog communities in some regions suffer chytridiomycosis die-offs immediately following *Bd* introduction (Lips 1999; Lips *et al.* 2003) and we now know that the virulence of *Bd* is highly temperature dependent (Piotrowski *et al.* 2004).

*Bd* is a diploid, single celled fungus with an infectious flagellated zoospore stage and a sessile thallus stage that produces reproductive bodies called zoosporangia (Longcore *et al.* 1999; Berger *et al.* 2005). Zoospores are free-living and will swim through water until encountering and encysting within amphibian skin (Longcore *et al.* 1999). New zoospores are produced clonally within each *Bd* zoosporangium and are released into the environment via discharge tubes that extend from the zoosporangium to the skin cell surface (Longcore *et al.* 1999). Sexual reproduction may rarely occur among *Bd* strains, but this process has never been confirmed, and low genetic diversity among worldwide *Bd* strains (Morgan *et al.* 2007) suggests that reproduction is generally
asexual. *Bd* only infects amphibian tissues containing keratin, which includes the outer skin layers of metamorphosed individuals (Longcore *et al*. 1999), and the mouthparts of tadpoles, which become deformed but do not lead to tadpole mortality (Rachowicz & Vredenburg 2004). The severity of disease is dependent on the amount of fungal growth (Vredenburg *et al*. 2010), and frogs with mild infections often show no signs or symptoms, while a high *Bd* load leads to skin swelling and shedding, redness, lethargy, seizures, and often death. Frogs have permeable skin that is important for respiration, hydration, and osmoregulation, and *Bd* causes mortality by severely disrupting electrolyte transport across the skin which leads to cardiac arrest (Voyles *et al*. 2009).

Temperature is the most important factor in determining *Bd* growth and pathogenicity. *Bd* grows optimally between 18-24°C and poorly at 10°C, but is killed in the laboratory when grown at temperatures above 28°C (Piotrowski *et al*. 2004). Similarly, *Bd* pathogenicity is highest at temperatures ranging from 12-23°C and lowest at temperatures above 27°C (Berger *et al*. 1998, 2004; Longcore *et al*. 1999; Carey *et al*. 2006). *Bd* survives across pH values ranging from 4 to 8, but growth is optimal at a pH of 6-7 (Piotrowski *et al*. 2004). Zoospores require moisture to survive, but some strains survived for 12 weeks in soil with only 10% moisture (Johnson and Speare 2005). Together, these findings present a wide range of natural environmental conditions in which *Bd* can flourish.

**Environmental Contributions**

*Bd* and chytridiomycosis prevalence are affected by numerous ecological and environmental variables. Studies of natural populations have shown *Bd* to vary with latitude (Kriger *et al*. 2007), elevation (Brem & Lips 2008), precipitation (Puschendorf *et al*. 2009, Longo *et al*. 2010), temperature (Kriger *et al*. 2007, Puschendorf *et al*. 2009), habitat loss (Becker & Zamudio 2011) and host species richness (Searle *et al*. 2011).
However, there is not a single epidemiological pattern for all geographic regions or host species. *Bd* infections occur in a wide variety of habitats, ranging from high elevations in the Sierra Nevadas of California (Vredenburg et al. 2010) to lowland forests of Gabon (Bell et al. 2011) and tropical rainforests of Australia (Berger et al. 1998).

Chytridiomycosis outbreaks are generally more frequent and severe in tropical compared to temperate environments (Lips et al. 2003). Disease is most prevalent in upland tropical habitats, where cool moist conditions favor rapid proliferation and survival of the fungus (Lips et al. 2003, Houlanhan et al. 2000). Roughly 70% of mid and high elevation tropical frog species are susceptible to chytridiomycosis, with over half of these going extinct (Lips et al. 2003; La Marca et al. 2005; Lips et al. 2006). The remaining species either decline and persist in low numbers (~20%) or are unaffected (~10%).

Temperate frog communities also experience disease outbreaks (Bradley et al. 2002; Green et al. 2003; Muths et al. 2003), but severity is often diminished due to seasonal abatement of *Bd* growth and/or pathogenicity (Kriger & Hero 2007a). The incidence and prevalence of *Bd* infections have been shown to be higher during cool months compared to warm months in numerous regions, including Australia (Retallick et al. 2004), Canada (Ouellet et al. 2005), the United States (Bradley et al. 2002), and Puerto Rico (Longo et al. 2010).

Taken together, studies of *Bd* across regions, habitats and seasonal conditions demonstrate that chytridiomycosis is a complex disease influenced not only by the environment, but by a unique combination of interactions among environmental variables. For example, increased habitat loss predicted lower disease risk in Australia, Brazil and Puerto Rico due to changes in host community structure and microclimate associated with habitat destruction (Becker & Zamudio 2011).
Host Contributions

Variation in host susceptibility to chytridiomycosis is the least explored corner of the Bd disease triangle. We know that chytridiomycosis decimates some amphibian species, while others in the same localities are seemingly unaffected (Retallick et al. 2004; Daszak et al. 2004; Lips et al. 2006). Interspecific studies show that certain species-level characteristics, including life history (Kriger & Hero 2007b) and innate (skin peptide) immune defenses (Woodhams et al. 2006; Woodhams et al. 2007), can partially predict which species are most at risk for chytridiomycosis. Intraspecific studies also demonstrate that, at least within some species, individuals vary in Bd susceptibility (Bradley et al. 2002; Kriger & Hero 2006). These findings suggest a role for host immune function in determining chytridiomycosis susceptibility, but this possibility remains underexplored. Experimental studies in the model frog genus Silurana show that under some conditions, Bd infection activates innate immune defenses (Ribas et al. 2009) or minimal immune responses of any kind (Rosenblum et al. 2009), while under other conditions, acquired immunity plays a role in host responses (Ramsey et al. 2010). Additionally, Major Histocompatibility Complex (MHC) immune regulatory genes were explored in a single species, Bufo calamita, and genotype frequencies among larval populations varied in a pattern consistent with directional selection in response to pathogen prevalence (May et al. 2011). Thus, amphibian immunogenetic resistance to Bd is a strong candidate mechanism for observed variation in susceptibility, but more functional and genetic studies are needed to confirm this pattern across host species, environmental conditions, and pathogen strains.

The Disease Triangle: Future Directions

To date, we have amassed an impressive body of literature on the epidemiology of Bd, from strain-specific differences in pathogenicity (Retallick & Miera 2007) to
complex models of pathogen dynamics under different environmental conditions (Briggs et al. 2010). However, poor knowledge of amphibian immunity is inhibiting our ability to fully elucidate disease dynamics in numerous host amphibians. Vertebrate immune systems are incredibly sophisticated and complex due to the necessity of having effective defense mechanisms for survival against the multitude of pathogenic organisms routinely encountered in the environment. Numerous immune pathways are likely to play a role in host responses to Bd, and rapid immunogenetic evolution may occur in response to strong selective pressure imposed by chytridiomycosis. To understand the extent, nature, and importance of these processes, further characterization of amphibian immune systems is sorely needed.

The best understood amphibian immune system is that of the model frog genus *Silurana* (Robert & Cohen 2011). Characterization of immune structure and function in *Silurana* reveals that amphibian immune systems are fundamentally similar to mammals (Du Pasquier et al. 1989). Frogs have a thymus where T cells differentiate and a spleen where B and T cells accumulate, and leukocytes such as neutrophils, basophils, eosinophils, monocytes, and macrophage-like cells are found in the blood (Robert & Ohta 2009). However, frogs lack lymph nodes, Peyer’s patches, and germinal centers, and consequently, frogs show poor affinity maturation, the process by which B cells make antibodies with increased affinity for antigen during the course of a mammalian immune response (Du Pasquier et al. 2000). Studies of *Silurana* provide the best evidence of immune system conservation and differentiation among vertebrate taxa, yet *Silurana* is polyploid, fully aquatic, and resistant to Bd, making the genus a biological novelty among amphibians. With the exception of antimicrobial peptides, which are well characterized across diverse anuran taxa and in the context of chytridiomycosis (Woodhams et al. 2006; Woodhams et al. 2007; Rollins-Smith et al. 2011), basic knowledge of amphibian immunity is lacking across non-model species. Lab and field
studies of immune responses in non-model amphibians infected with \textit{Bd} are thus the critical next steps in identifying host factors that may enhance survival in the face of chytridiomycosis.

The emergence of chytridiomycosis remains among the most serious threats to amphibian species worldwide. The 2008 Global Amphibian Assessment found that 42% of all amphibian species are declining, 159 species have likely gone extinct, and chytridiomycosis is the fastest growing threat to species persistence (IUCN 2008). Continued study of each corner of the chytridiomycosis disease triangle, with emphasis on host factors and their interaction with the pathogen and the environment, is our best approach for elucidating novel factors that promote \textit{Bd} survival and mitigate the impact of this global pandemic.
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CHAPTER 1:
DISEASE DYNAMICS VARY SPATIALLY AND TEMPORALLY IN A NORTH AMERICAN AMPHIBIAN†

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Abstract

Local environmental conditions are a primary factor influencing chytridiomycosis, an emerging disease caused by the fungus *Batrachochytrium dendrobatidis (Bd)* that has affected over 200 amphibian species worldwide. In North America, seasonality and regional habitat differences predict considerable spatial and temporal disease variability, yet the sparse and opportunistic nature of most studies have provided insufficient data for understanding regional *Bd* epidemiology. We present a five-year field study that reveals spatial and temporal *Bd* dynamics across *Lithobates yavapaiensis* populations in Arizona, USA. Two populations showed no *Bd* infection or mortality, ten populations showed winter *Bd* infection, and five populations experienced winter mortality. Infection intensity decreased over winter sampling seasons, whereas mortality and infection prevalence did not change over time. Frogs dying from chytridiomycosis were significantly larger and had significantly higher infection intensities than survivors. We

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conclude that conserving *L. yavapaiensis* and other native amphibians requires protection and management of riparian ecosystems to promote populations large enough to survive winter cycles of chytridiomycosis.

**Introduction**

*Batrachochytrium dendrobatidis* (*Bd*) is a fungal pathogen that has caused declines or extinction in over 200 amphibian species worldwide (Lips *et al.* 2006; Stuart *et al.* 2004). The consequences of *Bd* infection vary tremendously with local climatic conditions (Crawford *et al.* 2010; Kriger and Hero 2007; Longo *et al.* 2010). In upland tropical regions that experience little temperature variation across seasons, *Bd* infection often causes rapid declines in host amphibians from epidemics of the skin disease chytridiomycosis (Berger *et al.* 1998; La Marca *et al.* 2005). In contrast, amphibians in temperate regions with larger seasonal temperature fluctuations show variable responses to *Bd*, ranging from no observed effects (Garner *et al.* 2006; Longcore *et al.* 2007), to sub-lethal fitness costs (Retallick & Miera 2007), to episodic chytridiomycosis outbreaks and concomitant population declines (Kriger & Hero 2006; McDonald *et al.* 2005). These punctuated chytridiomycosis outbreaks usually occur in cool months, suggesting that cooler temperatures promote *Bd* growth, host susceptibility, or both.

With the exception of a few well-studied regions in the Sierra Nevada mountains (Briggs *et al.* 2010; Vredenburg *et al.* 2010), studies of local climatic determinants of chytridiomycosis in North America are patchy and conflicting. One retrospective study of North American museum specimens detected lower *Bd* prevalence in warmer months (Ouellet *et al.* 2005), while a similar contemporary study (Green *et al.* 2002) found chytridiomycosis outbreaks beginning in warm and cool months. In Canada, *Lithobates pipiens* *Bd* prevalence decreased as mean monthly temperatures increased (Voordouw *et al.* 2010), but survival did not differ among infected and uninfected individuals. In
Mexico, colder temperatures were associated with higher chytridiomycosis severity in four frog species (Hale et al. 2005). In contrast, Lips et al. (2004) documented chytridiomycosis mortalities in upland regions of Mexico during summer. These inconsistent patterns highlight the need to clarify the relationship between Bd infection, climate, local environmental conditions, and development of chytridiomycosis in North American amphibians.

Here, we present a 5-year field study of Bd infection dynamics in the lowland leopard frog (Lithobates [Rana] yavapaiensis), a stream-dwelling species inhabiting southwestern desert regions that has experienced chytridiomycosis die-offs since at least 1992 (Bradley et al. 2002). Documented chytridiomycosis outbreaks in L. yavapaiensis occur only in cooler months, but population surveys have been opportunistic (Sredl 2003). We conducted standardized, repeated surveys of twelve populations and measured Bd prevalence, intensity, and mortality in summer and winter. We used these data to determine whether (1) Bd infection and mortality vary among populations; (2) Bd infection and mortality vary seasonally; (3) Bd infection and mortality vary across years; and (4) Bd infection intensity, prevalence, and mortality co-vary within and across seasons, years and populations. Finally, we exploited the unique thermal dynamics in one region of Arizona to directly evaluate the relationship between water temperature, Bd infection, and chytridiomycosis.

Methods

We surveyed twelve L. yavapaiensis population localities in Arizona, USA, in summer (July–August) and winter (January–February) from 2006 to 2010 (Figure S1). All populations were surveyed within three weeks to limit within-season variation due to local environmental conditions. We gave equal survey time and effort across seasons, years, and localities, and used diurnal visual encounter surveys (Crump & Scott 1994) to
measure the abundance of adult and sub-adult *L. yavapaiensis* individuals. We toe-clipped and swabbed the epidermis of each individual using sterile fine-tip swabs (Medical Wire & Equipment Co. MW113) following standardized protocols (Hyatt *et al.* 2007). Frogs were handled with unused latex gloves to prevent pathogen transmission. Recapture rates were low (mean = 0.4%), thus we treated each sampling season as independent.

We used a Taylor 9842 temperature logger to record water temperatures. Within each locality, we took three mid-afternoon readings and retained the maximum recorded temperature. We separated Muleshoe Ranch (MR) into three sub-regions based on mean temperature variation across microhabitats: (1) MR<sub>HS</sub> is a thermal spring >50°C at the source and >30°C throughout the frog sampling region, (2) MR<sub>SS</sub> is a pond 0.5 km away fed by a thermal spring >30°C at the source and 20–30°C throughout the frog sampling region, and (3) MR<sub>BC</sub> is a canyon stream 1.4 km away that has average winter water temperatures of 10°C. To evaluate the role of water temperature, we separated frog populations into thermal spring localities (MR<sub>HS</sub> and MR<sub>SS</sub>) and non-thermal spring localities (all other populations).

Eight of our 12 localities are Nature Conservancy properties with continual monitoring since the 1980s by on-site staff (MR<sub>SS</sub>, MR<sub>HS</sub> MR<sub>BC</sub>, AC, HR), or sites that have been surveyed by Tucson Herpetological Society volunteers since the 1990s (AS, TV, CIC; Table S1.1). For each dead and dying frog, we recorded the date of death as the first date that our team, a volunteer, or a site manager observed the mortality event. We collected individuals with signs of chytridiomycosis (i.e., skin redness, lethargy, failure to seek cover, and loss of righting ability) for overnight observation; if death occurred within 24h and the individual tested positive for *Bd*, we categorized these frogs as chytridiomycosis mortalities. Individuals found dead during winter without other apparent causes of death (i.e., predation or injury) and testing positive for *Bd* were also
considered chytridiomycosis mortalities.

We extracted DNA from swabs using PrepMan Ultra (Applied Biosystems; Hyatt et al. 2007). Infection prevalence and intensity were determined using quantitative PCR (Boyle et al. 2004). Infection intensity was measured as the number of zoospore equivalents per swab, and individuals were considered *Bd*-positive at ≥1 zoospore equivalents. Values were not obtained for the January 2007 season. We calculated *Bd* infection as the number of positives divided by the total number of frogs swabbed and *Bd* mortality as the number of dead frogs divided by the total number of frogs observed. We calculated 95% Clopper-Pearson binomial confidence intervals from observed proportions of infection and mortality. We compared *Bd* infection, chytridiomycosis mortality, numbers of frogs, and water temperatures using two-tailed paired sample Wilcoxon signed-rank tests and rank sum tests. We compared *Bd* infection intensity among dead, dying and asymptomatic individuals using two-tailed Student’s *t*-tests assuming unequal variances.

**Results**

We sampled 692 *L. yavapaiensis* individuals from 12 localities in Arizona, USA, with a mean pairwise population distance of 198 km (Table S1.1). No mortality and low *Bd* prevalence (1.6%) occurred in summer. In contrast, all populations were infected with *Bd* in winter except for House Spring (HS) and Aliso Spring (AS; Figure 1.1A), populations separated by 265 km. Of the ten infected populations, five experienced winter mortalities, and observed levels of *Bd* infection and mortality varied among localities (Figure 1.1A). *Bd* infection (*P* = 0.005) and mortality (*P* = 0.02) were significantly lower in summer compared to winter. *Bd* infection was significantly higher than mortality in summer (*P* = 0.03) and winter (*P* = 0.008).
Figure 1.1: (A) Observed prevalence of winter *Lithobates yavapaiensis* *Bd* infection (closed symbols) and chytridiomycosis mortality (open symbols) across populations and years, with 95% Clopper-Pearson binomial confidence intervals indicated. Infection prevalence was measured as the proportion of swabbed individuals that harbored *Bd* infections, and chytridiomycosis mortality prevalence was measured as the proportion of frogs observed in winter that died with signs of chytridiomycosis. (B) Winter *Lithobates yavapaiensis* mean *Bd* infection prevalence (solid line) and chytridiomycosis mortality prevalence (dashed line) across sampling winters. Error bars show 95% Clopper-Pearson binomial confidence intervals. (C) Mean *Bd* infection intensity (measured as zoospore genome equivalents) across sampling winters. Error bars show standard
Winter Bd infection prevalence did not significantly change across sampling years, nor did winter Bd mortality (Figure 1.1B). In contrast, infection intensity significantly decreased from 2008 to 2010 ($P=0.02$; Figure 1.1C). Across all years, Bd infection intensity limits were 0–4040 zoospore equivalents among the 139 individuals sampled in winter months. Notably, we detected little Bd on individuals found dead; mean infection intensity was 1280.6 for dying individuals, 276.3 for healthy individuals, and 15.6 for individuals found dead. Dead individuals laid out for a mean of 5.3 days (range: 0-16) without predation before we collected them. Individuals found alive but showing signs of chytridiomycosis all died within 24 hours, and these moribund individuals had significantly higher mean infection intensity compared to individuals found dead ($P=0.02$). Individuals sampled from populations with compared to without mortality did not differ in mean infection intensity ($P=0.89$). Among individuals sampled in winter, mean body mass was significantly higher for dead (28.5 g) and dying (33.7 g) individuals compared to asymptomatic individuals (18.2 g; $P=0.01$). Bd infection prevalence was highly seasonal, increasing in winter and decreasing in summer for all populations across the 5-year sampling period (Figure 1.2A). Across all localities, the mean number of frogs observed was significantly higher in summers compared to winters ($P=0.001$). At Tanque Verde Canyon (TV), the locality with the highest infection and mortality prevalence (Figure 1.1A), we did not observe any frogs after summer 2009.
Figure 1.2: (A) Observed number of frogs (dashed line) and *Bd* infection prevalence (solid line) within each *Lithobates yavapaiensis* population in summer and winter from 2006 through 2010. (B) Mean maximum water temperature (filled circles), *Bd* infection prevalence (open circles), and chytridiomycosis mortality prevalence (open squares) for thermal spring (solid line; $N=2$) and non-thermal spring (dashed line; $N=10$) populations in winter versus summer.
We examined the effect of water temperature on *Bd* infection and chytridiomycosis mortality by comparing thermal springs to other localities. Among non-thermal spring localities, maximum water temperature was significantly higher in summer versus winter (*P*=0.002), whereas maximum water temperature at thermal springs did not differ significantly in summer versus winter (*P*=0.18; Figure 1.2B). In summer, maximum water temperature was not significantly different at thermal versus non-thermal spring sites (*P*=0.166). In contrast, maximum winter water temperature was significantly higher at thermal versus non-thermal spring sites (*P*=0.03; Figure 1.2B). Concordantly, chytridiomycosis mortality was significantly higher at non-thermal versus thermal spring localities in winter (*P*=0.05). However, *Bd* infection did not differ significantly at non-thermal spring versus thermal spring localities in winter (*P*=0.28) or summer (*P*=0.39).

**Discussion**

Seasonal and spatial variation in disease prevalence are common in human and wildlife systems (Hosseini *et al.* 2004; Pascual and Dobson 2005) and can arise from numerous factors, such as pathogen thermal requirements (Van Riper *et al.* 1986), host immunity changes over seasons or temperatures (Cheng *et al.* 2009), and variable host population genetic diversity (Pearman & Garner 2005). However, *Bd* modeling suggests that intraspecific variation in infection outcomes do not require differences in susceptibility, virulence, or environment, but can instead result from epidemic versus endemic dynamics of the same host-pathogen interaction (Briggs *et al.* 2010). Understanding the intraspecific dynamics of *Bd* in *L. yavapaiensis*, a species that continues to persist despite high chytridiomycosis susceptibility, may provide critical knowledge for the recovery of other, more susceptible species.

The higher infection and disease prevalence in cooler seasons we detected in *L.*
*yavapaiensis* is consistent with other studies of *Bd* in natural amphibian populations (Berger *et al.* 2004; Kriger & Hero 2006). Schlaepfer and colleagues (2007) estimated *Bd* infection prevalence in Arizona *L. yavapaiensis* from March through October and found no seasonal variation, no infection at three localities, and concluded *Bd* was likely excluded from these localities. We sampled two of the same three localities (MR, HS and HR) and found 33% and 37% winter *Bd* prevalence, respectively. This finding highlights the need for sampling protocols to cover the range of seasons and temperatures to assure that prevalence estimates are not biased.

Our study is the first to report *Bd* infection intensity in an amphibian of the southwestern United States, and we recovered two surprising patterns. First, we found that chytridiomycosis susceptible individuals were significantly larger and had significantly higher infection intensities than individuals that survived winters, contradicting other studies of *Bd* across age and size classes. For example, juvenile *Dendrobates tinctorius* were more likely to die from chytridiomycosis than adults (Lamirande & Nichols 2002), lower mass was associated with higher mortality in *Alytes obstetricans* (Garner *et al.* 2009), and metamorphs had significantly higher fungal loads than adults in *Lithobates sierrae* (Briggs *et al.* 2010). Very few *L. yavapaiensis* juveniles are captured in winter, thus our demographic infection patterns may result from the seasonal comparison of different sizes of adult frogs. In contrast, we saw no difference in infection between juveniles and adults during summer, thus we can exclude the possibility that juveniles emerging in the summer uninfected was skewing our summer prevalence estimates. The second surprising pattern was that we found low *Bd* intensities on frogs found dead, whereas dying frogs swabbed within 24h of death had the highest *Bd* loads. This pattern suggests that that other microbes displace *Bd* or that *Bd* zoospores disperse from frog carcasses after death, but to our knowledge these phenomena are undocumented in the literature. Alternately, some of the dead frogs we
found may have died from other causes; however, the fact that they were laying out in the open without signs of predation at localities where other frogs were dying of chytridiomycosis makes this explanation unlikely.

We found invariant *Bd* dynamics at thermal spring sites, where water perennially averages >30°C, suggesting that seasonal chytridiomycosis differences at all other Arizona localities result from cooler winter water temperatures. Analysis of disease dynamics throughout Australia found strong negative effects on *Bd* when air temperatures averaged >30°C (Drew *et al.* 2006), laboratory studies identify 30°C as the upper threshold for *Bd* viability (Piotrowski *et al.* 2004), and water temperature was negatively correlated with *Bd* infection intensity in North American newt populations (Raffel *et al.* 2011). Our study corroborates that temperature contributes to *Bd* infection outcomes, but we cannot confirm whether this pattern results from effects on the host, the pathogen, or both. Host immunity may play a role, as amphibian immune defenses decrease with temperature (Raffel *et al.* 2006) independent of season (Jozkowicz & Plytycz 1998), and in the laboratory, innate immune defenses against *Bd* occur at 26°C but not 18°C (Ribas *et al.* 2009). What remains uncertain is the relative importance of increased pathogen virulence compared to decreased host immune defenses under cooler environmental conditions.

In the United States, *Bd* infects over 50 amphibian species in the Southeast (Chatfield *et al.* 2009), Northeast (Hossack *et al.* 2010), Northwest (Pearl *et al.* 2007), and Rocky Mountains (Muths *et al.* 2008) with no reports of chytridiomycosis outbreaks. However, if die-offs occur during winter months when temperate-zone amphibians are inconspicuous and rarely monitored, chytridiomycosis declines may have gone unnoticed. The extreme seasonality of chytridiomycosis observed in *L. yavapaiensis* suggests that similar *Bd* dynamics may be occurring throughout North America. Consequently, better monitoring efforts should be in place for temperate-zone
amphibians that have experienced enigmatic declines, such as *Lithobates pipiens* (Lanoo 2005).

Amphibians of southwestern North America face a multitude of environmental threats (Witte *et al.* 2008). In Arizona and Mexico at least 13 native species are infected with *Bd* (Hale *et al.* 2005; Sredl 2003), and 36% of 324 known ranid frog populations became extirpated from Arizona during 1986–2001 (Witte *et al.* 2008). We focused on *L. yavapaiensis* because it remains widespread compared to congeners such as *L. chiricahuensis*, which has declined precipitously (Clarkson & Rorabaugh 1989), and *L. tarahumarae*, which has been extirpated from the United States (Hale & Jarchow 1988). However, chytridiomycosis continues to exert a toll on *L. yavapaiensis* populations, and habitat destruction and introductions of nonnative animals present additional threats (Witte *et al.* 2008). Our study population with the highest measures of winter mortality (TV) was possibly extirpated, highlighting the fragility of these populations in the face of disease. *Bd* infects numerous amphibian species and may survive in temperate aquatic systems outside of amphibian hosts (Walker *et al.* 2007) making pathogen eradication a difficult solution. Management for *Bd* should thus be added to eliminating other threats, such as bullfrogs, crayfish, non-native fish, and land use practices that increase erosion and alter pools required by *L. yavapaiensis* (Wallace *et al.* 2010), in order to promote populations large enough to persist through winter cycles of chytridiomycosis.

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CHAPTER 2:
GENETIC AND ENVIRONMENTAL PREDICTORS OF POPULATION RESPONSES TO
AN EMERGENT AMPHIBIAN PATHOGEN

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Abstract

A central aim of evolutionary biology is understanding how geography and environmental variation shape genetic differentiation in natural populations. The disease chytridiomycosis, caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*), has led to population die-offs in the lowland leopard frog (*Lithobates yavapaiensis*) since the 1970s, therefore this pathogen has potentially shaped genetic diversity among surviving populations. Here, we used microsatellite loci to characterize genetic differentiation in 11 *L. yavapaiensis* populations with variable disease dynamics. We estimated migration and explored the selective and demographic conditions that may permit populations to adapt to *Bd* at varying levels of gene flow. We found that two *Bd*-uninfected populations belonged to the same genetic deme, whereas each *Bd*-infected population was genetically unique. We detected one outlier locus that deviated from neutral expectations and had a signal of directional selection, indicating possible linkage with a genetic region under selection. Within populations, alleles of this outlier locus were significantly correlated with chytridiomycosis mortality among individuals. Across populations, neutral genetic diversity and environmental variables predicted *Bd* infection and mortality.
prevalence, whereas the outlier locus showed no significant patterns. We conclude that genetic isolation across a variable disease landscape in the presence of adaptive genetic diversity has permitted each population to independently evolve resistance to \textit{Bd}, despite similarities among populations in the environmental factors controlling pathogen growth and survival. Continued integration of genetic and environmental contributions to disease dynamics can help resolve \textit{Bd} spatial epidemiology and generate more effective conservation management strategies for mitigating global chytridiomycosis.

\textbf{Introduction}

Infectious diseases are potent agents of natural selection (Darwin 1871) that can impact population demography and have significant effects on population genetic variation, even at ecological timescales (Tishkoff & Verrelli 2003; Campbell \textit{et al.} 2010). Host-pathogen systems are particularly useful for studies of natural selection over short timescales because the potentially strong selective pressure imposed by some infectious agents has the potential to drive rapid host adaptation (Thrall & Jarosz 1994; Dwyer \textit{et al.} 1997; Duffy & Sivars-Becker 2007; Cavatorta \textit{et al.} 2008). While the genetic mechanisms of host resistance, host tolerance, and pathogen virulence are well known in human disease systems (Feng \textit{et al.} 2004; Råberg \textit{et al.} 2007; Barreiro & Quintana-Murci 2010) and in plants (Flor 1956; Fineblum & Rausher 1995; Qihan \textit{et al.} 2010), less is known about the genetic basis for evolving disease resistance in natural animal populations (but see Bernatchez & Landry 2003). One common pattern emerging from studies of natural populations is a positive correlation between host genetic diversity and resistance to disease (Meagher 1999; Zhu \textit{et al.} 2000; Pearman & Garner 2005), a pattern that has been attributed to higher adaptive potential in genetically diverse populations or species (Frankham 2005).
Chytridiomycosis is an emerging infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) that has caused population declines or extinction in over 200 amphibian species worldwide (Skerratt *et al.* 2007). In many tropical and subtropical regions, chytridiomycosis has spread in an epidemic wave (Lips *et al.* 2006; Cheng *et al.* 2010), causing sudden population and/or species extinctions (La Marca *et al.* 2005) and in some cases, becoming enzootic in host populations that persist (Murray *et al.* 2009; Longo & Burrowes 2010). In other regions, species are infected by *Bd* but have no known history of chytridiomycosis outbreaks (Lane *et al.* 2003; Garner *et al.* 2006; Longcore *et al.* 2007; Savage *et al.* 2011b), while others show intraspecific variation in *Bd* susceptibility among individuals and populations (Savage *et al.* 2011a; Tobler & Schmidt 2010; Kriger *et al.* 2007). These regional epidemiological differences have often been attributed to differences in climatic factors that affect *Bd* growth and/or persistence (Rohr *et al.* 2008). Although lab and field studies now confirm *Bd* dependence on environmental variables such as latitude, elevation, precipitation, and temperature (Carey *et al.* 2006; Kriger *et al.* 2007; Brem & Lips 2008; Rohr *et al.* 2011), few studies have examined host genetic variability underlying chytridiomycosis tolerance or resistance, and how the evolution of genetically resistant populations may occur in a climatically variable landscape. To date, genetic studies of host susceptibility focus on patterns of gene expression (Rosenblum *et al.* 2009) and variation in innate (Woodhams *et al.* 2006, 2007) or acquired (Savage & Zamudio 2011) immune genes, but those studies are either derived from laboratory experiments or do not explicitly consider population genetic changes that may have occurred during or after initial outbreaks of chytridiomycosis. Thus, to fully understand disease outcome in natural populations, we need more information about the distribution of genetic variability in natural populations, and how it relates to the evolutionary potential for disease resistance under naturally variable environmental conditions.
Identifying loci linked to adaptive phenotypic divergence presents a challenge in non-model species with limited genomic resources. However, recent approaches that focus on genetic divergence among natural populations have greatly improved our ability to detect markers under selection using a limited number of loci (Pritchard et al. 2000b; Beaumont & Balding 2004; Excoffier et al. 2009). Assuming that all loci have experienced the same demographic history, mutation rates, and migration among populations, locus-specific estimates of $F_{ST}$ can be used to identify genomic regions that have been under selection. Specifically, loci with exceptionally high levels of differentiation are more likely to have experienced directional selection, whereas loci with low levels of differentiation were likely constrained by stabilizing selection (Beaumont & Balding 2004; Holsinger & Weir 2009). Associations between environmental variables and allele frequencies often arise when the intensity of selection varies spatially across populations (Novembre & Di Rienzo 2009). We can therefore identify populations experiencing local adaptation by combining data from population disease variables and unlinked genetic markers and comparing them across populations. Chytridiomycosis is a primary cause of worldwide amphibian declines, thus identifying genetic regions potentially correlated with differences in $Bd$ exposure outcomes across populations would provide important insights into host responses to varying selective pressure imposed by this emergent pathogen.

Population genetic theory predicts that pathogens can both influence, and be influenced by, vertebrate host genetic diversity (Haldane 1949). However, while numerous studies document individual-level associations between infection and genetic polymorphism (Acevedo-Whitehouse et al. 2003; Ortego et al. 2007a), the impact of population level genetic diversity on pathogen dynamics remains unresolved. Some studies correlate host population genetic diversity to pathogen prevalence (Meagher 1999; Pearman & Garner, 2005). In contrast, other studies of host-pathogen systems
detect no relationship between genetic variability of the host and prevalence of the pathogen (Ortego et al. 2007b; Hawley et al. 2010). A simple relationship between host population genetics and pathogen dynamics may be unlikely, given the multitude of environmental factors that can influence disease in natural populations (Osnas & Lively 2004). Indeed, epidemiological researchers highlight the need for studies integrating genetic, spatial, and environmental processes influencing pathogen dynamics and host population genetics (Balkenhol et al. 2009; Biek & Real 2010).

The lowland leopard frog, *Lithobates yavapaiensis*, is a stream-dwelling species occurring in desert regions of southwestern North America. A recent range-wide phylogeography based on mitochondrial markers demonstrated that *L. yavapaiensis* populations expanded historically from northwestern Arizona into northern Mexico during the Pleistocene (Oláh-Hemmings et al. 2010). However, in recent decades *L. yavapaiensis* has experienced population declines and range contractions (Clarkson & Rorabaugh 1989; Scott 1993) due in part to chytridiomycosis outbreaks (Bradley et al. 2002; Savage et al. 2011a). Currently, *L. yavapaiensis* persists in the Sonoran Desert of Arizona, USA and Sonora, Mexico and in western New Mexico where it is nearly extirpated (Platz & Frost 1984; Jennings & Hayes 1994; Sredl 2005). The persistence of multiple isolated populations following dramatic chytridiomycosis declines in the 1990s (Bradley et al. 2002) and *Bd* infection since at least 1974 (Hale et al. 2005; Schlaepfer et al. 2007) indicates *Bd* resistance may have evolved in some populations, making this species an ideal candidate for quantifying the evolutionary genetic consequences of an emerging infectious disease.

In this study, we characterize population genetic structure among *L. yavapaiensis* populations and look for associations between genetic variation, environmental variation, and *Bd* disease dynamics. We previously described the prevalence and infection
intensity of \textit{Bd} in Arizona \textit{L. yavapaiensis} populations (Savage \textit{et al.} 2011a) and confirmed that chytridiomycosis varies spatially among populations and temporally among seasons, causing mortality only in winter. Here, we use winter \textit{Bd} infection and mortality estimates, multi-locus host genotypes, and a suite of environmental variables to explore the relationship between host, pathogen, and environment in shaping \textit{Bd} dynamics. We identify whether each genetic locus has a signature of neutral evolution or selection, and analyze each selective category separately. Within populations, where individuals face equivalent environmental regimes, we look for disease associations between individual genetic markers and \textit{Bd} mortality. At a unique thermal spring site with locally variable environmental regimes, we evaluate the evolutionary potential for local adaptation to \textit{Bd} between subpopulations in a case where gene flow counteracts distinct selective regimes. Finally, among populations, where individuals may face distinct environmental conditions, we consider the relative roles of host population genetics and environmental variables in predicting \textit{Bd} dynamics. Together, our analyses provide insight into past, present and future interactions of host population genetics with pathogen dynamics and environmental variation in a declining amphibian species.

\textbf{Methods}

\textit{Pathogen sampling}

We collected epidermal swabs from \textit{L. yavapaiensis} individuals at eleven localities in Arizona, USA, in winters (January – February) of 2006 through 2011 to characterize \textit{Bd} prevalence, \textit{Bd} infection intensity and \textit{Bd}-associated mortality at each of these localities (Savage \textit{et al.} 2011a). All samples included in this study were collected during winter months because chytridiomycosis outbreaks in Arizona \textit{L. yavapaiensis} populations occur during cool months when temperatures are pathogen-optimal (Savage \textit{et al.} 2011a). \textit{Bd} infection intensity, measured as the number of \textit{Bd} genome equivalents
per swab, was determined by extracting \textit{Bd} DNA from swabs with Prepman Ultra (Applied Biosystems) and using quantitative PCR (qPCR) methods developed for \textit{Bd} quantification (Hyatt \textit{et al.} 2007). We measured winter \textit{Bd} infection prevalence as the number of \textit{Bd} positive swabs divided by the total number of swabs collected (N=145), and \textit{Bd} mortality prevalence as the number of frogs found \textit{Bd} positive and dead or dying divided by the total number of frogs observed (N=483). We calculated 95\% Clopper-Pearson binomial confidence intervals for prevalence from the observed proportions of infection and mortality in each population sample.

The Muleshoe Ranch (MR) locality included three neighboring sub-populations with variable environmental conditions affecting disease prevalence: (1) Muleshoe Ranch Hot Spring (MR\textsubscript{HS}) is a thermal spring that is >50\(^\circ\text{C}\) at the source and >30\(^\circ\text{C}\) throughout the region where frogs were sampled, (2) Muleshoe Ranch Secret Spring (MR\textsubscript{SS}) is a nearby pond fed by a small thermal spring that is >30\(^\circ\text{C}\) at the source but below 30\(^\circ\text{C}\) elsewhere, and (3) Muleshoe Ranch Bass Canyon (MR\textsubscript{BC}) is a nearby canyon stream that is not fed by a thermal spring (Schlaepfer \textit{et al.} 2007). Frogs from MR\textsubscript{HS} and MR\textsubscript{SS} were \textit{Bd}-infected but demonstrated no winter chytridiomycosis mortality, whereas some frogs from MR\textsubscript{BC} were both \textit{Bd}-infected and died from chytridiomycosis in winter months (Savage \textit{et al.} 2011a). For purposes of analyses, the MR locality was therefore divided into the two populations, MR\textsubscript{HS/SS} and MR\textsubscript{BC}, for comparisons of disease status.

\textit{Host population sampling and microsatellite genotyping}

We collected toe tips from frogs at each of the 11 study populations (mean=46 \pm 21 individuals per site) in summers (July-August) and winters (January-February) of 2006-2010. In total we genotyped 509 individuals at fourteen unlinked microsatellite loci (Savage \\& Jaeger 2009), including all of the individuals swabbed in winter to infer \textit{Bd}
infection prevalence and intensity. Template DNA for microsatellite genotyping was extracted using a 5% Chelex 100 solution (Bio-Rad Laboratories) with 0.5 µg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. Supernatants from these extractions were used directly as template for PCR amplification, performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different fluorescent labels or non-overlapping size ranges were multiplexed and electrophoresed on a 3730 Genetic Analyser (Applied Biosystems). Fragments were sized by comparison to the LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems). We used MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) to test for the presence of scoring errors and null alleles.

**Population genetic structure and diversity**

We used STRUCTURE version 2.1 (Pritchard et al. 2000a) to identify the most likely number of genetic demes (K) present in our sample and the coefficient of membership to those demes for each *L. yavapaiensis* individual genotyped. STRUCTURE uses a Bayesian algorithm to cluster genotypes in the absence of any geographic information. We used a model allowing admixture and assuming uncorrelated gene frequencies, and ran 3,000,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in of 1,000,000 iterations. We assessed adequacy of run parameters by examining convergence of key summary statistics (Pritchard et al. 2000a). We conducted 20 independent runs for each value of K and retained the run with the highest likelihood, and then used the second-order rate of change to determine the most likely value of K (Evanno et al. 2005).

We used GENEPOP 3.4 (Raymond & Rousset, 1995) to calculate observed and
expected heterozygosities and test for deviations from Hardy-Weinberg equilibrium (HWE) at each locus and population locality using a Monte Carlo chain method (1000 dememorizations, 100 batches, 1000 iterations; Guo & Thompson 1992) and a Bonferroni correction for multiple tests for a table-wide significance level of $\alpha=0.05$ (adjusted $P=0.0002$). We estimated $D$, a measure of differentiation (Jost 2008), across all population pairs using Software for the Measurement of Genetic Diversity (SMOGD) version 1.2.5 (Crawford 2009). We used FSTAT 2.1 (Goudet 1995) to test for linkage disequilibrium at each locus over all populations (adjusted $P=0.00004$) and to compute pairwise $F_{ST}$ values and their significance among localities (adjusted $P=0.0005$). We calculated genetic diversity indices for each population using the program GENALEX version 6 (Peakall & Smouse 2006).

*Inter-population migration and the potential for adaptive immunity*  
We characterized migration among populations using BAYESASS version 1.3 (Wilson & Rannala 2003), which uses Bayesian analysis and MCMC to estimate mean immigration rates among populations and their confidence intervals (CI). We assumed negligible drift over the last two generations, and that immigrants in a population did not exceed 33% per generation. Further, loci were assumed to be in linkage equilibrium and free of null alleles. We performed 9,000,000 iterations (following a 1,000,000 burn-in) and sampled every 2000 iterations.

To explore the dynamics of gene flow, drift and selection among neighboring populations differing in disease epidemiology, we focused on one locality, the Muleshoe Ranch (MR), where populations with different disease dynamics occur in close proximity and migration could thus potentially have large effects on the potential evolution of *Bd* resistance. We followed the model employed by Adkison (1995) and McCairns & Bernatchez (2008) to define the necessary demographic conditions leading to adaptive
divergence among the MR populations $\text{MR}_\text{HS}$ and $\text{MR}_\text{BC}$. The model is based on a numerical approximation of Slatkin’s (1973) characteristic length scale of variation in gene frequency ($l_c$), which defines the minimal cline distance at which populations cannot respond to environmental variation. Predictions of three alternative scenarios – genetic homogenization (H), differentiation due to random drift (R), or adaptive divergence (A) – are based on two derived variables: $\beta$, the ratio of migration to drift, and $k$, the ratio of the geographical scale at which selection favors a given allele ($j$) relative to $l_c$ (Nagylaki & Lucier 1980). Given that $\text{MR}_\text{HS}$ and $\text{MR}_\text{BC}$ fall into different disease categories, we took the stringent view that these localities represent independent populations with different selection acting in each ($j=1$). We used a range of estimates of effective population size ($N_e$) and strength of selection ($s$) to infer the conditions likely to lead to adaptive divergence (A: $\beta>1.1; k>1.1$), random differentiation (R: $\beta<1$), or genetic homogeneity (H: $\beta>1.1; k<1$) across the 95% confidence interval of estimated migration among $\text{MR}_\text{HS}$ and $\text{MR}_\text{BC}$.

**Loci under selection**

Loci that deviate from neutral evolutionary expectations, hereafter termed ‘outlier’ loci, potentially carry a signature of natural selection. We detected outlier loci in our study taxon using the Beaumont & Nichols (1996) Fdist approach implemented in LOSITAN (Antao et al. 2008). This method is based on the theoretical expectation that, for a range of population structures and demographic histories, genetic differentiation among populations in contrasting environments should be different for loci under selection when compared to neutral loci. LOSITAN uses coalescent simulations to generate a null distribution of $F_{ST}$ values based on an infinite island model, and loci with unusually high $F_{ST}$ values are putatively under directional selection, while loci with low $F_{ST}$ values are potentially under stabilizing selection. We simulated the neutral $F_{ST}$
distribution with 100,000 iterations and used a conservative significance threshold of $P < 0.005$. We then calculated all pairwise population $F_{ST}$ values and compared them to population heterozygosity measures to identify $F_{ST}$ outliers. Runs were performed using two possible mutation models: the stepwise mutation model, most commonly applied to microsatellite markers (Valdés et al. 1993) and the infinite allele model.

**Genetic predictors of disease within populations**

We tested for associations between individual microsatellite genotypes and chytridiomycosis susceptibility among *Bd*-infected *L. yavapaiensis* individuals using STRAT (STRuctured population Association Test; Pritchard et al. 2000b). STRAT controls for spurious disease-allele associations that arise due to the presence of common alleles with unequal frequencies across genetic groups by examining disease associations independently for each genetic deme identified using Structure (Pritchard et al. 2000a). The test statistic $\Lambda$ represents the likelihood of association between allele frequencies and disease phenotype within genetic groups. We assigned disease phenotypes as 0 if the individual was found alive without disease signs, and 1 if the individual was found dead or dying. *Bd* mortality (i.e., dead infected frogs) is an unambiguous assignment, whereas frogs sampled alive could potentially have died at future time points, therefore this analysis is designed to detect genetic associations with *Bd* susceptibility, but not *Bd* resistance. We generated $\Lambda$ for each of the 14 microsatellite loci and inferred significance probabilities by comparison to 10,000 random simulations of genotype frequencies within genetic groups.

**Environmental and genetic predictors of disease across populations**

We used Generalized Linear Models (GLMs) to test for associations of disease variables (*Bd* mortality prevalence, infection prevalence, and infection intensity) with
genetic and environmental variables across populations. For environmental variables, we obtained canopy density information from Global Forest Resource Assessment [1000m resolution; coverage period 1995-1996 (USGS-FAO 2000)] and elevation data (90m resolution) from CGIAR Consortium for Spatial Information (Jarvis et al. 2009). Additionally, nineteen bioclimatic variables were extracted using Worldclim/Bioclim layers (1000m resolution) available at <http://www.worldclim.org/bioclim> (Hijmans et al. 2005). These metrics of temperature and precipitation are averaged from fifty-year records (1950-2000) from a dense network of climatic stations throughout the world (e.g. precipitation records from 47,554 locations, temperature from 24,542 locations). All environmental variables were measured at a 1km diameter buffer from sampling locations to maintain a consistent scale across all variables in the analyses. We also included maximum water temperature for each site, averaged across winter field surveys (3-5 measurements per visit). We used ArcGIS 9.3.1 in all analyses (ESRI 2009). For genetic variables, we used GENALEX version 6 (Peakall & Smouse 2006) to calculate population measures of observed heterozygosity, expected heterozygosity, mean number of effective alleles, and mean number of private alleles for the 13 neutral loci combined and for the outlier locus (C110).

We identified important genetic factors predicting each of the response variables (Bd mortality prevalence, infection prevalence, and infection intensity) using linear regressions. To test for an association between genetic factors and both Bd mortality prevalence and Bd infection prevalence, we used GLMs with binomial distribution and logit link. To test for an association between genetic factors and Bd infection intensity we used GLMs with normal distribution and identity link. After this first univariate assessment, we used stepwise regressions (forward method; cutoff P < 0.20) to screen for environmental factors that potentially predict each of the response variables to be included in model selection procedures. For each response variable,
we screened a total of 23 explanatory variables including canopy density, latitude, elevation, mean winter maximum temperature, and the 19 bioclimatic temperature and precipitation metrics. We confirmed lack of spatial autocorrelation within each response variable using Moran’s I correlograms (Rangel et al. 2010). We used GLM model selections including environmental and genetic variables to explain each response variable and tested all possible models without interactions. Competing models were ranked based on Akaike Information Criterion (AICc), and we report the best-fit model for each run.

Results

Bd infection and chytridiomycosis mortality in L. yavapaiensis

We collected tissue samples and skin swabs from 509 L. yavapaiensis individuals from 11 localities in Arizona, USA (Figure 2.1). Observed levels of Bd infection and chytridiomycosis mortality vary significantly within and among these localities in winter months (Savage et al. 2011a). Our focal populations occupy a narrow range of elevations, and variation in elevation did not explain the observed disease patterns (Table S1.1). All populations were infected with Bd in winter months except for House Spring (HS) and Aliso Spring (AS). Of the infected populations, five experienced winter chytridiomycosis mortalities and five did not (Figure 2.2A). Mean Bd infection intensity was not significantly different between populations with and without winter mortality (Figure 2.2B). It is therefore unclear whether adaptation to Bd in L. yavapaiensis occurs via a mechanism of disease resistance (limiting pathogen burden) or disease tolerance (limiting the damaging effects of a given pathogen burden; Råberg et al. 2007), and mechanisms of survival may be variable across populations. For clarity, we hereafter refer to any process of surviving Bd infection as Bd resistance, although we are aware that tolerance may play a role.
Figure 2.1: Sampled *Lithobates yavapaiensis* populations in Arizona, USA. White symbols denote *Bd* uninfected populations, black symbols denote *Bd* infected populations, single circles denote populations without mortality, and double circles denote populations with mortality. The asterisk marks the location of the Muleshoe Ranch thermal spring population. Locality abbreviations are as follows: AS = Aliso
Spring; AC = Aravaipa Canyon; CC = Cottonwood Canyon; CIC = Cienega Creek; HS = House Spring; HR = Hassayampa River; MR = Muleshoe Ranch; SM = Santa Maria River; SS = Seven Springs; TV = Tanque Verde Canyon; WC = Willow Creek.

Table 2.1: Pairwise estimates of $F_{ST}$ (upper) and $D$ (lower) for each collection locality. All values are significant except those in italics.

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<td>0.335</td>
<td>0.268</td>
<td>0.604</td>
</tr>
<tr>
<td>AC</td>
<td>0.713</td>
<td>–</td>
<td>0.315</td>
<td>0.318</td>
<td>0.237</td>
<td>0.161</td>
<td>0.269</td>
<td>0.301</td>
<td>0.244</td>
<td>0.275</td>
<td>0.328</td>
</tr>
<tr>
<td>TV</td>
<td>0.825</td>
<td>0.541</td>
<td>–</td>
<td>0.393</td>
<td>0.248</td>
<td>0.275</td>
<td>0.405</td>
<td>0.479</td>
<td>0.406</td>
<td>0.386</td>
<td>0.483</td>
</tr>
<tr>
<td>AS</td>
<td>0.891</td>
<td>0.674</td>
<td>0.500</td>
<td>–</td>
<td>0.275</td>
<td>0.239</td>
<td>0.312</td>
<td>0.395</td>
<td>0.336</td>
<td>0.348</td>
<td>0.412</td>
</tr>
<tr>
<td>CIC</td>
<td>0.785</td>
<td>0.526</td>
<td>0.337</td>
<td>0.507</td>
<td>–</td>
<td>0.178</td>
<td>0.312</td>
<td>0.356</td>
<td>0.335</td>
<td>0.299</td>
<td>0.357</td>
</tr>
<tr>
<td>MR_{BC}</td>
<td>0.764</td>
<td>0.427</td>
<td>0.502</td>
<td>0.534</td>
<td>0.447</td>
<td>–</td>
<td>0.253</td>
<td>0.256</td>
<td>0.257</td>
<td>0.247</td>
<td>0.328</td>
</tr>
<tr>
<td>SM</td>
<td>0.749</td>
<td>0.735</td>
<td>0.859</td>
<td>0.764</td>
<td>0.764</td>
<td>0.699</td>
<td>–</td>
<td>0.204</td>
<td>0.159</td>
<td>0.221</td>
<td>0.356</td>
</tr>
<tr>
<td>WC</td>
<td>0.755</td>
<td>0.773</td>
<td>0.885</td>
<td>0.844</td>
<td>0.816</td>
<td>0.634</td>
<td>0.492</td>
<td>–</td>
<td>0.180</td>
<td>0.227</td>
<td>0.452</td>
</tr>
<tr>
<td>HR</td>
<td>0.608</td>
<td>0.597</td>
<td>0.804</td>
<td>0.776</td>
<td>0.825</td>
<td>0.684</td>
<td>0.414</td>
<td>0.501</td>
<td>–</td>
<td>0.194</td>
<td>0.392</td>
</tr>
<tr>
<td>SS</td>
<td>0.505</td>
<td>0.719</td>
<td>0.784</td>
<td>0.838</td>
<td>0.729</td>
<td>0.674</td>
<td>0.610</td>
<td>0.624</td>
<td>0.571</td>
<td>–</td>
<td>0.366</td>
</tr>
<tr>
<td>HS</td>
<td>0.869</td>
<td>0.640</td>
<td>0.600</td>
<td>0.556</td>
<td>0.597</td>
<td>0.687</td>
<td>0.779</td>
<td>0.840</td>
<td>0.817</td>
<td>0.732</td>
<td>–</td>
</tr>
</tbody>
</table>

Genetic diversity and differentiation by disease category

Our 14 microsatellite markers were highly polymorphic across sampled populations (Figure 2.3). The total number of alleles per locus ranged from 10 to 33 with a mean value of 19; within populations, the mean number of alleles ranged from 2.7 to 10.0 (Figure 2.3). Nine populations had at least one locus with a significant deviation from HWE, but no locus showed deviation in a majority of populations (Table S2.2), thus we included all data in further analyses. Pairwise estimates of $F_{ST}$ and Jost’s $D$ indicated high genetic differentiation among all populations except for the three neighboring MR populations (Table 2.1). Excluding comparisons among the MR populations, mean
pairwise $F_{ST}$ was 0.32 (range 0.17-0.60) with 90% of comparisons significant (adjusted $P = 0.000549$) and mean $D$ was 0.64 (range 0.41-0.91) with all comparisons significant (95% confidence intervals did not include zero).

**Figure 2.2:** (A) *Lithobates yavapaiensis* observed winter *Bd* infection (closed symbols) and chytridiomycosis mortality (open symbols) by locality, with 95% Clopper-Pearson binomial confidence intervals. (B) Logarithm of mean population winter *Bd* infection intensity measured as the average number of genome equivalents recovered per animal.
The two *Bd* uninfected populations had lower heterozygosity, number of alleles, and number of effective alleles compared to all other populations (Figure 2.3). *Bd* tolerant populations (SM, HR, SS, MR) showed higher diversity, number of alleles, and number of effective alleles than *Bd* susceptible populations (MR, CIC, CC, AC, TV, WC); however, across *Bd* susceptible populations these measures varied considerably. Few private alleles were detected in any population, but MR, AC, and SM had the highest values (Figure 2.3).

**Figure 2.3:** Patterns of allelic richness and heterozygosity in 11 sampled populations of *Lithobates yavapaiensis* genotyped at 14 microsatellite loci. Bars represent mean ± SD number of alleles (white bars), mean ± SD number of effective alleles (grey bars), and mean ± SD number of private alleles (black bars). Mean ± SD heterozygosities for each population (across all loci) are represented by the black line. Populations are arranged according to *Bd* infection dynamics, ranging from no infection or mortality to infection only to infection with mortality.
Structure analyses revealed 10 genetic clusters or demes that corresponded to the 11 geographic sampling localities with one exception: individuals from localities House Spring (HS) and Aliso Spring (AS) were assigned to the same genetic deme despite the large geographic distance separating these populations (265 km) and the presence of four genetically distinct populations in the intervening region (Figure 2.4). Notably, these two populations were assigned to the same genetic deme for values of $K$ ranging from 6-12, indicating a strong signal of genetic ancestry. Individuals from all other geographic populations were assigned to independent genetic demes with an average $q$ of 0.89 (range 0.74-0.95). Hereafter, locality names will thus refer to both genetic and geographic populations, with the label HS/AS for the House Spring/Aliso Spring shared deme.

**Figure 2.4:** Structure analysis results for $K = 10$ with individuals grouped by locality and disease status. Vertical bars show the proportion of membership to each genetic deme for 509 *Lithobates yavapaiensis* individuals genotyped at 14 microsatellite loci. Population abbreviations follow Figure 2.1. The 10 genetic demes are represented as follows: AS/HS = purple; MR = light pink; SM = light blue; HR = red; SS = pink; CC = dark green; AC = light green; TV = yellow; CIC = blue; WC = tan.
Migration and the potential for adaptive immunity

BAYESASS estimates indicated negligible contemporary migration \( (m) \) among all pairs of populations \( (m = 0-6\% \text{ immigrant ancestry}, \text{mean } m = 0.02\%, \text{all values non-significant}) \) except among the sub-populations within the MR locality (Figure 2.5; Table S2.3). Migration among these sub-populations was considerable, statistically significant, and unidirectional from the thermal springs locality (MR\(_{\text{HS}}\)) into both the pond (MR\(_{\text{SS}}\); \( m = 27\% \text{ immigrant ancestry from MR}_{\text{HS}} \)) and the canyon (MR\(_{\text{BC}}\); \( m = 21\% \text{ immigrant ancestry from MR}_{\text{HS}} \)). Migration was not detected between MR\(_{\text{SS}}\) and MR\(_{\text{BC}}\), or from either of these localities back to MR\(_{\text{HS}}\).

We modeled the potential for the MR\(_{\text{BC}}\) subpopulation to evolve chytridiomycosis resistance given the high migration rate from the thermal springs, which environmentally shelters frogs from chytridiomycosis due to perennially high temperatures unfavorable to \( \textit{Bd} \) (Savage \textit{et al.} 2011a; Schlaepfer \textit{et al.} 2007). We parameterized adaptive divergence models with a broad range of effective population sizes \( (N_e) \) ranging from 10-10,000 individuals, and included migration rates spanning the 95\% confidence interval of estimated dispersal from MR\(_{\text{HS}}\) into MR\(_{\text{BC}}\) (14 – 29\% per generation). Assuming that \( \textit{Bd} \)-imposed selection is zero at the thermal springs, the model predicts that the strength of selection \( (s; \text{range } 0-1) \) for chytridiomycosis resistance in the canyon must be greater than 0.07 for the MR\(_{\text{BC}}\) frogs to adapt at the lower 95\% CI of migration, independent of \( N_e \) (Table 2.2). If migration rates are closer to the estimated mean value of 21\%, \( s \) must be greater than 0.11 for the canyon frogs to evolve \( \textit{Bd} \) resistance, regardless of \( N_e \).
Table 2.2: Predicted values of effective population size ($N_e$) and strength of selection ($s$) leading to adaptive divergence (A), genetic homogenization (H), or random differentiation (R) across the 95% confidence interval range of migration rates ($m$) estimated from site MRHS into site MRBC. Predictions are based on the model of Nagylaki & Lucier (1980).

<table>
<thead>
<tr>
<th>$N_e$</th>
<th>$m$</th>
<th>$s = 0.001 - 0.07$</th>
<th>$s = 0.071 - 0.10$</th>
<th>$s = 0.11 - 1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.14</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>100</td>
<td>0.14</td>
<td>H</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1000</td>
<td>0.14</td>
<td>H</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>10,000</td>
<td>0.14</td>
<td>H</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>100,000</td>
<td>0.14</td>
<td>H</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>0.21</td>
<td>R</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>100</td>
<td>0.21</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>1000</td>
<td>0.21</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>10,000</td>
<td>0.21</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>0.29</td>
<td>R</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>100</td>
<td>0.29</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>1000</td>
<td>0.29</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>10,000</td>
<td>0.29</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>100,000</td>
<td>0.29</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
</tbody>
</table>

Detection of a locus under selection and disease associations within populations

The LOSITAN $F_{ST}$ analysis identified one of the 14 microsatellite loci as an outlier in 121 pairwise comparisons ($P < 0.005$). Locus RoC110 was found to be under directional selection with an exceptionally high $F_{ST}$ value (Figure 2.6A). All other loci fell within the expected range for neutrally evolving genetic markers.

The 10 genetic demes identified using Structure were further analyzed to test for associations between microsatellite allele frequencies and two chytridiomycosis phenotypes: (1) alive, meaning the individual was observed to be alive and healthy regardless of $Bd$-infection status, or (2) dead, meaning the individual was $Bd$-infected and found dead or dying with signs of chytridiomycosis. Locus RoC110 showed a significant association between allele frequencies and $Bd$ infection phenotype within
genetic groups ($\lambda=21.09$, df=11, $P=0.009$). In populations with $Bd$ mortality, allele frequency distributions for this locus were significantly different between dying and surviving individuals (Figure 2.6B). However, the same alleles were not associated with chytridiomycosis phenotype across different genetic demes.

![Diagram of Muleshoe Ranch subpopulations](image)

**Figure 2.5**: BAYESASS estimates of migration ($m$) among Muleshoe Ranch (MR) *Lithobates yavapaiensis* subpopulations ($N$=sample size). Open circles indicate sampling from localities with $Bd$ infection but no chytridiomycosis mortality. Filled circles indicate sampling from localities with $Bd$ infection and chytridiomycosis mortality. Arrows show the presence and direction of migration. For each subpopulation, 95% confidence intervals for the proportion of individuals with immigrant ancestry are indicated in parentheses.
Genetic and environmental predictors of disease

Among all population genetic measurements, the best predictors of population disease status were observed heterozygosity and number of private alleles for the neutral loci, and observed heterozygosity for the outlier locus. For the neutral loci, observed heterozygosity significantly predicted mortality prevalence ($\beta = -9.699, \chi^2 = 19.067, P < 0.001$), but was not associated to $Bd$ infection prevalence ($\beta = -1.951, \chi^2 = -0.733, P = 0.392$) or $Bd$ infection intensity ($\beta = 2.735, \chi^2 = 0.309, P = 0.578$). We also found a positive association between number of private alleles, $Bd$ infection prevalence ($\beta = 1.620, \chi^2 = 11.463, P < 0.001$), and $Bd$ infection intensity ($\beta = 0.693, \chi^2 = 0.354, P = 0.552$), but not $Bd$ mortality prevalence ($\beta = 0.410, \chi^2 = 0.736, P = 0.391$). For the outlier locus, we did not find significant associations of heterozygosity with mortality prevalence ($\beta = 1.240, \chi^2 = 1.827, P = 0.176$) or $Bd$ infection intensity ($\beta = 1.053, \chi^2 = 0.205, P = 0.650$), although we found a marginal association with $Bd$ infection prevalence ($\beta = 1.737, \chi^2 = 3.745, P = 0.053$).

Our initial stepwise screening for meaningful environmental variables selected four variables to explain mortality prevalence, two variables to explain $Bd$ infection prevalence, and eight variables to explain $Bd$ infection intensity. When jointly considering these effects in a model selection approach including all possible models, genetic factors remained as strong predictors of $Bd$ infection prevalence and mortality prevalence, but not $Bd$ infection intensity (Table S2.4). Among the best models, $Bd$ mortality prevalence had a strong negative association with neutral observed heterozygosity, mean diurnal temperature range, and annual precipitation (Table 2.3). Likewise, $Bd$ infection prevalence had a strong negative association with neutral observed heterozygosity. In the same model, the number of private alleles and minimum temperature of the coldest month positively predicted $Bd$ infection intensity (Table 2.3). None of the genetic factors were included in the best models predicting $Bd$
infection intensity (Table S2.4). In the most likely model, three temperature and rainfall metrics were the best explanatory variables (Table 2.3).

**Table 2.3:** Generalized Linear Models simultaneously testing the effects of genetic and environmental factors on Bd mortality prevalence, infection prevalence, and infection intensity among 11 populations of *L. yavapaiensis* in Arizona.

**Bd Mortality Prevalence**

<table>
<thead>
<tr>
<th>Term</th>
<th>$\beta$</th>
<th>SE</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>64.121</td>
<td>14.197</td>
<td>48.016</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Observed Heterozygosity (Ho)</td>
<td>-13.335</td>
<td>2.839</td>
<td>27.772</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean Diurnal Temperature Range</td>
<td>-0.324</td>
<td>0.072</td>
<td>46.067</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Annual Precipitation</td>
<td>-0.157</td>
<td>0.006</td>
<td>10.929</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Bd Infection Prevalence**

<table>
<thead>
<tr>
<th>Term</th>
<th>$\beta$</th>
<th>SE</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>5.031</td>
<td>1.854</td>
<td>8.379</td>
<td>0.004</td>
</tr>
<tr>
<td>Observed Heterozygosity (Ho)</td>
<td>12.441</td>
<td>4.073</td>
<td>11.077</td>
<td>0.001</td>
</tr>
<tr>
<td>Number of Private Alleles</td>
<td>3.355</td>
<td>0.780</td>
<td>25.197</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Min Temperature of Coldest Month</td>
<td>0.060</td>
<td>0.017</td>
<td>20.161</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Bd Infection Intensity**

<table>
<thead>
<tr>
<th>Term</th>
<th>$\beta$</th>
<th>SE</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>35.323</td>
<td>3.075</td>
<td>28.205</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temperature Annual Range Precipitation of Wettest Month</td>
<td>-0.072</td>
<td>0.007</td>
<td>24.597</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Precipitation of Driest Quarter</td>
<td>-0.089</td>
<td>0.006</td>
<td>31.858</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Whole model tests: mortality prevalence ($\chi^2 = 65.328$, DF = 3, $P < 0.001$); Bd infection prevalence ($\chi^2 = 44.767$, DF = 3, $P < 0.001$); Bd infection intensity ($\chi^2 = 34.719$, DF = 3, $P < 0.001$). Final models chosen based on AICc.
Figure 2.6: (A) $F_{ST}$ versus heterozygosity for fourteen microsatellite loci. The dark gray area shows the upper 99.5% confidence interval and the white area shows the lower 99.5% confidence interval with 10,000 simulations from the stepwise mutation model. (B) Allele frequencies of the outlier locus RoC110 that showed significant associations with Bd mortality within genetic demes. Numbers of individuals and allele frequencies among alive and dead *L. yavapaiensis* individuals are shown for the five genetic demes that experience *Bd*-associated mortality. Each shade of grey represents a different allele.
Discussion

Host population genetics and infectious diseases interact spatially and temporally, producing epidemiological patterns that can both shape current host population structure and be shaped by historical host population demography (Boots et al. 2004, Biek et al. 2006). Our study demonstrates the utility of combining measures of disease dynamics with environmental variables and genetic differentiation at neutral and outlier loci to disentangle the effects of environment, demography, gene flow, and selection on Bd epidemiological patterns among populations. We identified an outlier locus and compared it with neutral loci, permitting the detection of spatial genetic correlations with disease variables while excluding historical, demographic, or other population processes. To date this approach has been used infrequently (Luikart et al. 2008; Gebremedhin et al. 2009; Richter-Boix et al. 2011), but it has immense potential to elucidate genetic adaptation in locally varying selective environments (Lowry 2010). Our study is also the first, to our knowledge, that models genetic and environmental chytridiomycosis covariates simultaneously, revealing that host population genetics are significant predictors of disease dynamics when considering the entire selective, genetic, and environmental landscape.

Population structure and disease

Pairwise differentiation measures, genetic clustering analysis, and gene flow estimates each demonstrated that L. yavapaiensis populations are highly genetically differentiated. This extensive genetic structure differs from a recent mitochondrial phylogeographic study that showed all L. yavapaiensis populations except one belonged to a nearly undifferentiated group with haplotypes varying at most by two nucleotides (Oláh-Hemmings et al. 2010). This discordance is expected, because microsatellite markers with more rapid mutation rates, reflect more recent demographic processes,
and offer the resolution to infer the limits to gene flow in this system. The number and size of *L. yavapaiensis* populations have declined considerably in the last half-century due to severe fragmentation, alteration of aquatic habitats, introduced species, and chytridiomycosis (Sredl 2005). While the mitochondrial analysis revealed a strong signature of species expansion thousands of years before any of these factors were present, our results indicate that recent fragmentation and declines may have resulted in the observed loss of genetic connectivity among populations.

Structure analyses revealed a common genetic background for *Bd* uninfected populations, in stark contrast to the distinct genetic background for each *Bd* infected population (Figure 2.4). Membership to the same genetic deme for the two populations (HS and AS) that have likely never been exposed to *Bd* may arise due to a common genetic signature of *L. yavapaiensis* population structure in the absence of *Bd*. However, these populations also had the lowest measures of heterozygosity, number of alleles, and effective number of alleles (Figure 2.3), and both have exceptionally small population sizes (Savage *et al.* 2011a). Thus, isolation and drift are the likely forces shaping the distribution of genetic differentiation in these two populations, and further identification and sampling of *Bd*-uninfected populations will be necessary to determine the precise effects of the presence or absence of *Bd* infection on *L. yavapaiensis* genetic structure. Due to the current lack of migration and genetic distinctiveness of each *Bd* infected population, we conclude that *L. yavapaiensis* populations with *Bd* are evolving independently and each may adapt to disease pressure in novel and unique ways.

**Disease dynamics among sub-populations**

A recent review of associations between genetic variation and fitness traits across 34 amphibian studies found that genetic-fitness correlations were documented in 65% of cases (Allentoft & O’Brien 2010). In contrast, a second review focusing on longer-term
studies across organisms with good understanding of the traits under selection indicated that selection may exhaust genetic variance, thereby limiting a host’s response (Blows & Hoffmann 2005). Both reviews concluded that determining the extent of and constraints on genetic-fitness correlations would require more knowledge of the ecological settings and genetic background of natural populations than is currently available for most systems.

We exploited the environmental variation present at Muleshoe Ranch (MR) due to the thermal spring (Savage et al. 2011a) to dissect the role of fine-scale host evolutionary genetic processes versus environmental variation in constraining or allowing populations to adapt to Bd. The thermal spring water temperatures at MR_HS and MR_SS exceed the limits for optimal Bd growth (Piotrowski et al. 2004; Schlaepfer et al. 2007) or pathogenicity (Carey et al. 2006), whereas MR_BC winter water temperatures are cool enough to promote Bd proliferation (Savage et al. 2011a). All three MR subpopulations were infected with Bd but experienced distinct selective pressures due to this environmental variation. We predicted that MR_BC individuals were under selection for chytridiomycosis resistance, while the other subpopulations were environmentally sheltered from this selective pressure. However, MR_BC belongs to the same genetic deme as MR_HS and MR_SS and was not significantly differentiated based on pairwise $F_{ST}$ and $D$ values. This lack of differentiation occurred because 14-29% of the MR_BC subpopulation consisted of first- or second-generation immigrants from MR_HS (Figure 2.5). Modeling the parameters of migration, drift, and selection necessary to create this scenario showed that the selection coefficient imposed by Bd would need to be high ($s > 0.11$) for MR_BC frogs to adapt given the annual influx of susceptible genotypes from MR_HS. A selection coefficient of 0.11 corresponds to an 11% increase in fitness of individuals with the advantageous genotype when compared to individuals lacking that genotype. In genome-wide studies of polymorphisms in Drosophila, estimated selection
coefficients for single mutations range from 0.000012 to 0.02 (Jenson et al. 2008). In studies of human HIV, a highly virulent pathogen that imposes strong selection for resistance, whole-genome analyses detect selection coefficients for positively selected genotypes ranging from 8.0E−3 (Neher & Leitner 2010) to 0.09 (Liu et al. 2002). Thus, the threshold of 0.11 for MRBC frogs is unlikely to be met, even when selection for chytridiomycosis resistance is strong. Because the evolution of host resistance is most likely a fitness trade-off (Anderson & May 1982) and MRBC frogs only face punctuated selection for disease resistance in cooler months when Bd is most virulent (Carey et al. 2006), it is unlikely that selection will be strong enough for this population to become fixed for resistance to chytridiomycosis.

**Disease associations within populations**

We found one candidate locus, RoC110, that was an $F_{ST}$ outlier and showed significant allelic associations to Bd mortality within genetic groups. Comparing allele frequencies among dead and live frogs showed that this pattern is largely explained by mortality within population CIC. In this population, sample sizes were large enough to determine that a significant proportion of dead frogs had an allele that was never found in live frogs (Figure 2.6B, black fill). Notably, this allele was nearly fixed in dead and live frogs from population MR, the locality where gene flow and environmental sheltering likely overwhelms selection and prevents sub-populations from adapting to Bd. This allele was also nearly fixed in population TV, a locality with high winter Bd mortality that has possibly been extirpated since 2009 (Savage et al. 2011a). Taken together, these patterns suggest that within-population Bd susceptibility may include a functional genetic component linked to allelic variation at locus RoC110.

Molecular adaptation to local ecological or environmental factors (including pathogens) is commonly explored in model species or non-model taxa with extensive
genomic resources (De La Vega et al. 2002). In contrast, genetic signatures of adaptation are rarely explored in wildlife populations, likely due to the limited number of molecular markers available. Indeed, for traits under weak selection or for quantitative traits determined by multiple loci with small effects, significant patterns are unlikely to be detected from a limited set of loci. However, as the strength of selection on a given trait increases, so does the extent of linkage disequilibrium (Lewontin & Kojima 1960; Schork 2002). Thus, for populations facing strong selective pressure – for example, from a disease such as chytridiomycosis that causes massive population die-offs – selection may be sufficiently strong to create genetic associations detectable by a smaller number of unlinked genomic markers. Indeed, the ‘local-effects’ hypothesis, in which genotype-fitness associations result from a physical association between a neutral marker and a locus under selection (David 1998), has gained recent empirical support from studies showing that natural populations can show high levels of linkage disequilibrium (Yan et al. 1999; Reich et al. 2001) and that some loci contribute more than others to fitness associations (Hanson et al. 2004; Acevedo-Whitehouse et al. 2006).

The functional genetic region linked to RoC110 that may be responsible for producing significant associations with disease dynamics is not known; however, we suspect an immunity gene or haplotype, which could have large enough effects on survival to generate broad linkage disequilibrium with a microsatellite marker. Given the modest sample sizes and number of loci used in our study, further work is necessary to determine whether a functional genetic basis exists for the disease associations we detected. However, even if this association is ultimately determined to have a non-selective basis, the separation of purportedly neutral loci into outlier and neutral-conforming categories that are analyzed separately is critical for accurate assessment of neutral population structure, especially when genetic differentiation is inferred from a
limited number of loci.

**Disease associations among populations**

Several studies of *Bd* disease dynamics have analyzed ecological and environmental predictors of disease (Briggs *et al.* 2010, Becker & Zamudio 2011, Rohr *et al.* 2011), while other studies have identified genetic-fitness associations (May *et al.* 2011, Savage & Zamudio 2011). To our knowledge, this study is the first to jointly consider environmental and genetic factors contributing to *Bd* dynamics. Of all genetic factors tested, observed heterozygosity showed the strongest effect on population disease measures when also considering environmental factors, with higher heterozygosity predicting significantly lower *Bd* infection and mortality prevalence. This pattern is consistent with the ‘general effect’ hypothesis (David 1998), where heterozygosity across multiple microsatellite markers reflects genome-wide heterozygosity and is thus an indirect measurement of a population’s average fitness. In contrast, the outlier locus showed no significant patterns with disease across populations when also considering environmental factors, an expected result given that intra-population associations will likely be erased when only considering inter-population genetic measurements. Interestingly, the mean number of private alleles showed a positive relationship with *Bd* infection prevalence, suggesting that a higher proportion of unique alleles increases a population’s disease risk. This pattern likely occurs because populations with more private alleles tend to be smaller, more isolated populations with higher genetic drift (Slatkin & Takahata 1985) that are less able to genetically adapt to *Bd*. Finally, our analyses show that *Bd* infection intensity is completely environmentally determined, with no host genetic contribution. This result is concordant with the high variation we observed in mean infection intensities across populations; environmental conditions dictate *Bd* growth and therefore the magnitude of infection, but some
populations tolerate high infection intensities with no apparent consequences (e.g., HR) while others show mortality at fairly low infection intensities (e.g., WC). Thus, the functional consequence of infection intensity is locality-specific and should be compared within rather than among *L. yavapaiensis* populations.

In summary, we found a single outlier locus that showed significant association with *Bd* mortality within populations, whereas only neutral measures of heterozygosity and genetic drift were significant predictors of *Bd* mortality across populations. This pattern is consistent with the lack of gene flow we detected among sampled populations; because populations are completely isolated, they evolve as independent units based upon standing measures of genetic diversity and, in the case of the outlier locus, the presence and frequency of susceptibility-associated alleles at the time of initial *Bd* emergence. Our analyses of the Muleshoe Ranch hot spring and adjacent populations highlight the possible alternative outcomes if gene flow continued to unite all populations; selection against *Bd* susceptibility would need to be strong to overwhelm the effects of migration and drift, given that environmental conditions vary across populations and significantly contribute to *Bd* dynamics.

**Conclusions and conservation implications**

In this study, we demonstrated the utility of integrating genetic analyses of differentiation at neutral and outlier loci, individual disease assays, and environmental variables in natural wildlife populations to better understand potential evolutionary responses to disease pressure within and among populations. Our findings may prove useful in predicting the fate of populations facing pathogen selective pressure and designating important evolutionary lineages containing genetic variation for local adaptation to chytridiomycosis. *Bd* currently infects amphibians on every continent where they occur (Lips 1999, Bosch *et al.* 2001, Waldman *et al.* 2001, Hopkins &
Channing 2003, Ron et al. 2003, Green et al. 2003, Goka et al. 2009), and chytridiomycosis threatens the persistence of numerous species worldwide (Lips et al. 2006, Skerratt et al. 2007). Understanding the precise ecological and evolutionary dynamics that allow or prevent populations from persisting with Bd will be critical for accurate planning and implementation of species conservation efforts (Woodhams et al. 2011). For populations with the potential to evolve Bd resistance, this may entail management actions that promote genetic diversity and increase effective population sizes. Paradoxically, the dramatic fragmentation and isolation of L. yavapaiensis populations in recent decades (Witte et al. 2008) has prevented ongoing gene flow from erasing local adaptation to variable disease dynamics, heightening the evolutionary potential for remaining populations to overcome chytridiomycosis susceptibility. Preserving and promoting genetic diversity is therefore likely to be the most effective management strategy to increase the long-term probability of L. yavapaiensis species persistence in the face of ongoing chytridiomycosis outbreaks.

**Acknowledgements:** We thank the Zamudio lab and three anonymous reviewers for constructive comments on earlier versions of the manuscript; D. Caldwell, A. Martin, M. Booth, J. Richmond, M. Schlaepfer, R. Rogers, M. Lawrence and M. Haberstich for assistance in the field; and Steve Bogdanowicz, the Evolutionary Genetics Core Facility, and the Computational Biology Service Unit for help with data collection and analyses. This work was supported by a National Science Foundation (NSF) Graduate Research Fellowship, a National Geographic Society Young Explorers Grant, and an NSF Doctoral Dissertation Improvement Grant (number) to AES, and an NSF Population Evolutionary Processes grant (DEB-0815315) to KZ.
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CHAPTER 3:

MHC GENOTYPES ASSOCIATE WITH RESISTANCE TO A FROG-KILLING FUNGUS†

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Abstract

The emerging amphibian disease chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Amphibian populations and species differ in susceptibility to *Bd*, yet we know surprisingly little about the genetic basis of this natural variation. Major Histocompatibility Complex (MHC) loci encode peptides that initiate acquired immunity in vertebrates, making them likely candidates for determining disease susceptibility. However, MHC genes have never been characterized in the context of chytridiomycosis. Here, we performed experimental *Bd* infections in lab-reared frogs collected from five populations that show natural variation in *Bd* susceptibility. We found that alleles of an expressed MHC class IIB locus associate with survival following *Bd* infection. Across populations, MHC heterozygosity was a significant predictor of survival. Within populations, MHC heterozygotes and individuals bearing MHC allele Q had a significantly reduced risk of death, and we detected a significant signal of positive selection along the evolutionary lineage leading to allele Q. Our findings demonstrate that immunogenetic variation affects chytridiomycosis survival under controlled conditions.

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experimental conditions, confirming for the first time that host genetic polymorphisms contribute to chytridiomycosis resistance.

Introduction

Amphibians face a biodiversity crisis (Stuart et al. 2004) brought on in large part by the emerging infectious disease chytridiomycosis (Lips et al. 2006, Skerratt et al. 2007; Wake & Vredenburg 2008; Rohr et al. 2008). The fungal pathogen Batrachochytrium dendrobatidis (Bd) causes chytridiomycosis in amphibian species worldwide, but the extent of morbidity and mortality varies within and among populations (Lips 1998; Bradley et al. 2002), species (Lips 1998), and assemblages (Lips 1998; Crawford et al. 2010). Some of this variation is attributable to regional and local ecological factors such as climate (Pounds et al. 2006), elevation (Lips et al. 2006), host life history traits (Lips et al. 2003), and different Bd strains (Retallick & Miera 2007). However, host immunity likely also contributes to disease outcomes. In the model frog Silurana tropicalis, experimental Bd inoculations significantly change host gene expression profiles (Rosenblum et al. 2009), and innate (Ribas et al. 2009) and acquired (Ramsey et al. 2010) immune responses are activated at host-optimal temperatures. In addition, experimental infections of the toad Alytes obstetricans show that Bd survival varies among individuals and populations under constant environmental conditions (Tobler & Schmidt 2010), and upon reinfection, previously Bd-infected Leiopelma archeyi clear the pathogen significantly faster than control animals (Shaw et al. 2010), suggesting a secondary acquired immune response. Identifying heritable determinants of Bd resistance has the potential to enhance the success of amphibian conservation efforts (Woodhams et al. 2011). However, to date immunogenetic correlates of survival remain underexplored (Richmond et al. 2009).

Vertebrate Major Histocompatibility Complex (MHC) genes are good candidates
for influencing host Bd dynamics because they encode cell-surface glycoproteins that regulate the vertebrate acquired immune response and affect the development and progression of wildlife disease (Bernatchez & Landry 2003). To initiate acquired immunity, MHC proteins bind pathogen molecules on their peptide-binding regions (PBRs) and present them to T-cells (Jones et al. 2006). This central function in pathogen defense creates strong diversifying selection within host populations for numerous MHC proteins that can collectively bind a wide array of pathogens (Hughes & Yeager 1998).

MHC genes are divided into three classes (I, II and III) that are genomically linked across tetrapods (Ohta et al. 2006). Class I loci are expressed on all nucleated somatic cells and present peptides derived from intracellular pathogens, whereas class II genes are expressed in epithelial cells in the thymus and antigen-presenting cells in the periphery, and primarily present peptides derived from extracellular pathogens (Kaufman et al. 1985; Braciale et al. 1987). The class III region encodes a group of structurally unrelated proteins that are not involved in antigen presentation (but have immune function; Kaufman et al. 1985). Class II MHC loci are the most likely candidate immunity genes for chytridiomycosis for two reasons: (i) they are the primary presenters of extracellular fungal pathogens (Braciale et al. 1987) and (ii) class II-expressing dendritic and Langerhans lymphocytes are present in amphibian skin (Carillo-Farga et al. 1990; Du Pasquier & Flajnik 1990), the primary location of Bd infections. In spite of these data, class II genes have only been described in a few amphibians (Kaufman et al. 1985; Sato et al. 1993; Sammut et al. 1999; Liu et al. 2002; Bos & DeWoody 2005; Hauswaldt et al. 2007; May et al. 2011) and never in the context of Bd susceptibility.

Associations between disease susceptibility and MHC polymorphisms are widespread in natural wildlife populations (Bernatchez & Landry 2003). Most studies focus on variation in the peptide-binding region (PBR) of class I and class IIB loci because PBR amino acid variability determines the repertoire of pathogen peptides to
which T-cells can respond (Jones et al. 2006). Across diverse vertebrate taxa, survival after disease exposure has been associated with particular PBR alleles (Langefors et al. 2001), with elevated PBR heterozygosity (Penn et al. 2002), and with intermediate levels of PBR diversity (Wegner et al. 2003). These multiple mechanisms of disease resistance indicate that MHC-associated immunity is context-dependent and varies with each combination of host, pathogen, and environment. Chytridiomycosis is caused by a single pathogen affecting numerous amphibian hosts that occupy diverse habitats, thereby providing a unique epidemiological combination for immunogenetic studies of independent hosts evolving under varied environmental regimes.

The lowland leopard frog (Lithobates yavapaiensis) is a North American amphibian that began experiencing mass chytridiomycosis die-offs in the early 1990s (Bradley et al. 2002). Currently, populations persist with Bd infections that vary in prevalence and intensity across space and time (Savage et al. 2011, Schlaepfer et al. 2007). In some populations, a proportion of individuals die from chytridiomycosis during winter months when temperatures are pathogen-optimal (Savage et al. 2011). In other populations, Bd infection prevalence and intensity increase during winter months, but host frogs never develop signs of disease (Savage et al. 2011). This pattern suggests two non-exclusive hypotheses: (i) genetic resistance to chytridiomycosis occurs in some populations, or (ii) environmental variation explains differences in disease dynamics among populations. However, the spatial and seasonal variation in pathogen and disease occurrence across populations precludes distinguishing genetic from environmental determinants of Bd-associated mortality in the field.

Here, we determined the role of host genetic variation in Bd-associated mortality by experimentally infecting lab-reared L. yavapaiensis collected from five geographically distinct populations that naturally differ in disease dynamics. We exposed individuals to Bd and induced chytridiomycosis under constant, pathogen-optimal environmental
conditions. We then tested the following four hypotheses: (i) lab survival varies across individuals and/or populations in patterns that correspond to observed chytridiomycosis mortality in the field; (ii) pathogen burdens are lower in surviving individuals compared to individuals that die; (iii) genetic variation at MHC loci associates with survival following infection; and (iv) MHC alleles associated with survival show a signature of positive selection. Combined, these hypotheses address the overarching question of whether heritable host immune factors explain differences in \textit{Bd} susceptibility in natural populations.

\textbf{Results}

We characterized natural chytridiomycosis in five \textit{L. yavapaiensis} populations during 2007–2010 (Figure 3.1A; Figure S3.1; Savage \textit{et al.} 2011) to generate \textit{a priori} expectations for survival patterns in our experimental \textit{Bd} challenges. We collected egg masses in 2008 and reared them through metamorphosis in a light, temperature, and humidity controlled growth chamber. Prior to the experiment we tested for, but did not detect, native \textit{Bd} infections among our experimental animals. Metamorphosed frogs were randomly assigned to two replicated treatments: (i) infected with Arizona \textit{Bd} strain PsTr2004 via exposure to $1 \times 10^5$ zoospores in liquid media, or (ii) kept as uninfected controls exposed to liquid media only. Among the 99 frogs experimentally infected with \textit{Bd}, 14 individuals survived the experiment (Figure 3.1B; Table S3.1); survivors were from Aravaipa Canyon (AC) and the Santa Maria River (SM). These two populations showed 41\% and 27\% survival in the laboratory, respectively, and are also the two populations with the lowest proportion of mortalities in the field (Figure 3.1A). No experimentally infected frogs from Cienega Creek (CIC), Muleshoe Ranch (MR), or Willow Creek (WC) survived, corroborating the hypothesis that lab survival varies across populations according to chytridiomycosis mortality in the field. Mean infection intensity,
defined as the number of *Bd* genome equivalents recovered per swab, was not significantly different from zero at one (*P*=0.74) or three (*P*=0.39) days post infection (DPI), but zoospore counts increased significantly in all populations at seven DPI (*P*=0.0005; Figure 3.1C-D). At 14 DPI, mean infection intensity was no longer significantly different from zero for individuals from AC and SM that survived (*P*=0.5; open symbols, Figure 3.1D) but remained significantly higher than zero for all individuals that eventually died (*P*<0.0001; closed symbols, Figure 3.1D). Thus, among *Bd*-infected individuals, those that died maintained *Bd* infection intensities higher than zero for the duration of the experiment, whereas surviving individuals cleared *Bd* infections by 14 DPI (Figure 3.1C-D).

Population genetic background can bias disease association analyses (Pritchard *et al.* 2000a), therefore we genotyped all 99 *Bd*-infected frogs at 14 unlinked microsatellite loci (Savage & Jaeger 2009) to characterize population structure for comparison with immunogenetic variation. We implemented a Bayesian assignment test (Pritchard *et al.* 2000b) that confirmed the independent evolutionary history and current genetic isolation of each sampled population: frogs were assigned to six genetic demes (lnL = −2984.1) corresponding to their five natal populations, with further differentiation between clutches from population CIC (Figure 3.2A). We next characterized the PBR of an expressed MHC class IIB gene (Kiemnec-Tyburczy *et al.* 2010) and sequenced PBR genotypes for all *Bd*-infected individuals to test for associations between allelic polymorphisms and responses to *Bd* infection. We recovered 33 unique PBR alleles (GenBank accession nos. JN638850–JN638882; Figure S3), of which only six occurred in multiple genetic demes. Ten of the 12 sampled clutches showed evidence of multiple paternity based on microsatellite and PBR genotypes (Figure S3.2; Table S3.1), thus the majority of clutches included half-sibships, rather than full sibships.
**Figure 3.1:** *Bd* infection and mortality vary among populations of *Lithobates yavapaiensis* in nature and in the laboratory. (A) Field estimates of winter *Bd* infection prevalence (proportion of frogs testing positive for *Bd*; filled symbols) and mortality (proportion of *Bd*-positive frogs that died; open symbols) in five natural populations. AC = Aravaipa Canyon; CIC = Cienega Creek; MR = Muleshoe Ranch; SM = Santa Maria River; WC = Willow Creek; 95% Clopper-Pearson binomial confidence intervals are indicated (modified from 37). (B) Survival following *Bd* infection varies among individuals from the same five populations when reared in a growth chamber. Survival was tracked for 35 days post infection (DPI) with $1 \times 10^5$ zoospores of *Bd* (colored lines) or sham infection (black line). Population abbreviations follow (A); CIC1 and CIC2 denote genetically distinct clutches from population CIC. Numbers of individuals per population (*Bd*-infected/uninfected) are: AC, 17/11; CIC, 34/22; MR, 5/2; WC, 17/11; SM, 26/15; all, 99/59. (C) Mean *Bd* infection intensity (zoospore equivalents ± SD) measured at various days post infection (DPI) in populations with no survival (CIC, MR and WC). (D) Mean *Bd* infection intensity over time for survivors (open symbols; AC$_S$ and SM$_S$) and non-survivors (filled symbols; AC$_D$ and SM$_D$) from populations with survivors (AC and SM). Control frogs (black symbols) remained uninfected for the duration of the experiment.

To identify parameters with significant effects on survival, we used the Cox proportional hazard model including treatment, genetic deme, clutch, replicate, number of frogs per replicate, initial mass, change in mass, PBR heterozygosity, maximum infection intensity (MII), and interactions between MII and all other parameters (complete model: $\Delta \ln L=36.2$, df=22, $P<0.0001$). As expected, significantly more uninfected individuals survived compared to *Bd*-infected individuals ($P=0.001$). Among infected individuals, PBR heterozygosity ($P=0.0002$), MII ($P=0.005$), and the interaction between
MII and PBR heterozygosity ($P=0.0005$) were significant explanatory variables predicting survival. In contrast, density-dependent dynamics within replicates did not influence survival, as the number of frogs per replicate and the interaction between number of frogs per replicate and MII were not significant.

We generated an unrooted genealogy of unique PBR sequences to examine the distribution of alleles in relation to genetic demes and disease outcomes (Figure 3.2B). PBR alleles did not cluster by genetic deme. Of the alleles recovered from multiple populations (A–E and Q), frogs with alleles B and Q showed a significantly reduced risk of mortality (Table 3.1). This pattern could arise because alleles B and Q occur only within AC and SM, the two genetic demes in which some individuals survived. Thus, we tested for allelic associations within each of those demes, and found a significant reduction in risk associated with allele Q in SM but not AC (Table 3.1). Similarly, SM (signed-rank test, $P=0.03$), but not AC ($P=0.09$), showed significantly different PBR allele frequency distributions between survivors and non-survivors (Figure 3.3A). Across populations, PBR heterozygotes had a significantly reduced risk of death (Table 3.1; Figure 3.3C). Within populations, risk of death remained significantly reduced for heterozygotes in demes AC and SM (Table 3.1, Figure 3.3B), demonstrating that heterozygote advantage was not an artifact of higher heterozygote frequency in demes with survivors.
Figure 3.2: (A) Clutch genetic structure inferred by Bayesian assignment of 99 Bd-infected *L. yavapaiensis* individuals. Thin bars separate individuals, thick bars separate clutches, and asterisks indicate individuals that survived experimental infection. Horizontal bars show the coefficient of membership across genetic demes for each Bd-infected frog, with each color representing a unique genetic deme. (B) Maximum
likelihood genealogy of 33 MHC class IIB PBR alleles. Circle size is proportional to frequency, colors correspond to the genetic demes in (A), alleles recovered from survivors are shown with open symbols, and alleles recovered from non-survivors are shown with filled symbols. Bold denotes terminal branches with significantly elevated nonsynonymous substitution rates ($\omega > 1$) followed by the codon positions under selection in parentheses. Alleles showing significant associations with survival are enlarged and italicized. Bootstrap values >50% are indicated.

**Table 3.1:** Relative Risk (RR) for survival-associated MHC risk factors among *Bd*-infected frogs. 95% confidence intervals are in parentheses, followed by two-tailed Fisher exact test $P$ values. Significant RR values after sequential Bonferroni correction are shown in bold.

<table>
<thead>
<tr>
<th>Genetic deme</th>
<th>Risk Factor</th>
<th>Heterozygote</th>
<th>Allele B</th>
<th>Allele Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$0.13$</td>
<td>$0.46$</td>
<td>$0.0$</td>
</tr>
<tr>
<td>$AC$</td>
<td></td>
<td>$(0.13 – 0.43)$</td>
<td>$(0.14 – 1.2)$</td>
<td>$(0.0 – 1.3)$</td>
</tr>
<tr>
<td>$N=17$</td>
<td></td>
<td>$P = 0.001$</td>
<td>$P = 0.16$</td>
<td>$P = 0.41$</td>
</tr>
<tr>
<td>$SM$</td>
<td>$0.43$</td>
<td>$(0.32 – 0.77)$</td>
<td>$(0.26 – 1.2)$</td>
<td>$0.0$</td>
</tr>
<tr>
<td>$N=26$</td>
<td></td>
<td>$P = 0.005$</td>
<td>$P = 0.31$</td>
<td>$(0.0 – 0.71)$</td>
</tr>
<tr>
<td>$All$</td>
<td>$0.58$</td>
<td>$(0.54 – 0.78)$</td>
<td>$(0.27 – 0.74)$</td>
<td>$0.0$</td>
</tr>
<tr>
<td>$individuals$</td>
<td></td>
<td>$P &lt; 0.00001$</td>
<td>$P = 0.001$</td>
<td>$(0.0 – 0.55)$</td>
</tr>
<tr>
<td>$N=99$</td>
<td></td>
<td></td>
<td></td>
<td>$P &lt; 0.00001$</td>
</tr>
</tbody>
</table>
To distinguish between selection and neutral evolution, we estimated $\omega$, the ratio of nonsynonymous (amino acid-changing) to synonymous (silent) nucleotide substitutions. Values of $\omega$ equal to 1 indicate neutral evolution, whereas values greater than 1 indicate positive selection and values less than 1 indicate purifying selection (Kimura 1977). Along the 82 PBR codons, models including positive selection (allowing $\omega > 1$ as a rate class) fit the alignment significantly better than models excluding positive selection (Table 3.2). We detected significant positive selection in codons 3, 18 and 46, which correspond to three of 14 codon positions that determine peptide binding in the human class II PBR (Tong et al. 2006). These substitution rate analyses detect selection over the entire history of allelic lineages (Li et al. 1985) and do not distinguish among ancient versus recent selective events. Thus, to identify PBR alleles that may have recently fixed nonsynonymous substitutions in response to chytridiomycosis, we tested for positive selection ($\omega > 1$) along terminal branches of the genealogy, which represent the most recent molecular changes. We detected $\omega > 1$ in two terminal branches (Figure 3.2B, bold; Table S3.2). Codons 3 and 18 showed $\omega > 1$ along the branch leading to an allele present in an individual that died, thus we conclude this signal of selection is unrelated to $Bd$. In contrast, codon position 46 showed $\omega > 1$ along the branch leading to allele Q, the only allele recovered exclusively from surviving individuals (Figure 3.2B). Codon 46 may therefore be particularly important in determining $Bd$ peptide binding, and allele Q is a candidate allele for recent immunogenetic adaptation in response to disease pressure.
Table 3.2: Evidence of positive selection among 82 amino acid residues of *Lithobates yavapaiensis* MHC class IIB PBR sequences. \(dN−dS\) = normalized difference between nonsynonymous substitutions per nonsynonymous sites (\(dN\)) and synonymous substitutions per synonymous sites (\(dS\)); \(\omega = dN/dS\) ratio; PP = posterior probability.

<table>
<thead>
<tr>
<th>Rate class method</th>
<th>Likelihood ratio test ((P\text{-value}))</th>
<th>Percentage of sites in (\omega&gt;1) rate classes</th>
<th>Mean (\omega) per (\omega&gt;1) rate class</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARRIS</td>
<td>9.16 ((0.01))</td>
<td>15%</td>
<td>4.3</td>
</tr>
<tr>
<td>Evolutionary Fingerprinting</td>
<td>26.1 ((0.006))</td>
<td>27%</td>
<td>4.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Codon-specific method</th>
<th>Codon 3 (dN−dS) ((P\text{-value}/PP))</th>
<th>Codon 18 (dN−dS) ((P\text{-value}/PP))</th>
<th>Codon 46 (dN−dS) ((P\text{-value}/PP))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLAC</td>
<td>18.1 ((0.09))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEL</td>
<td>1.1 ((0.03))</td>
<td>0.93 ((0.03))</td>
<td>0.74 ((0.04))</td>
</tr>
<tr>
<td>REL</td>
<td>3.2 ((0.99))</td>
<td>3.3 ((0.99))</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3: MHC class IIB PBR allele and heterozygote frequencies for *Bd*-infected survivors and non-survivors. PBR allele distribution (A) and PBR heterozygote and homozygote frequency (B) among survivors (open bars) and non-survivors (filled bars) in AC (red bars) and SM (blue bars), the two genetic demes with survivors. Allele counts and numbers of heterozygous and homozygous individuals are indicated above frequency bars. (C) Survival curves for PBR heterozygotes (solid line) and homozygotes (dotted line) across *Bd*-infected individuals from all five genetic demes.

Discussion

We have shown that MHC class IIB-associated genetic factors explain differences in *Bd* susceptibility in *L. yavapaiensis* by confirming our four hypotheses: (i) *Bd* survival was significantly different among individuals and populations, and was concordant with observed variation in field mortality (Figure 3.1A-B), (ii) maximum pathogen burdens were significantly lower in surviving individuals (Figure 3.1C-D), (iii)
MHC heterozygotes and MHC allele Q showed significant associations with survival (Table 3.1; Figure 3.2B); and (iv) allele Q had a signal of recent positive selection acting on codon 46 (Figure 3.2B). Thus, MHC class IIB genotypes are significantly associated with differences in *Bd* survival in *L. yavapaiensis* populations. Our results should be interpreted with the caveat that the MHC contains numerous immune-related loci that are in strong linkage disequilibrium (Ohta *et al.* 2006), and MHC loci experience frequent and diverse forms of selection for pathogen resistance, self and non-self discrimination, and kin selection (Bernatchez & Landry 2003; Apanius *et al.* 1997). These varied evolutionary processes present a challenge for identifying specific effects of individual MHC genes. Indeed, we cannot exclude the possibility that the class IIB locus examined here shows significant associations with survival due to linkage with another locus, because at least two class II genes are expressed in *L. yavapaiensis* (Kiemnec-Tyburczy *et al.* 2010). However, the signal of positive selection we detected along the PBR lineage leading to the only allele significantly associated with *Bd* survival challenges linkage as the sole explanation for our results. To the contrary, recent positive selection acting in the peptide-binding region of a resistance-associated allele found in natural populations provides compelling evidence that this locus encodes a molecule with specific involvement in the immunological response to *Bd*.

Our results underscore the evolutionary advantage of diverse genetic resistance pathways in an ever-changing environment. Lowland leopard frogs show spatial and temporal variation in demography and *Bd* exposure (Savage *et al.* 2011), which likely creates population-specific selective regimes for the evolution of chytridiomycosis resistance. Furthermore, host *Bd*-resistance alleles may carry some fitness cost with regard to other *Bd* strains or pathogens (Apanius *et al.* 1997; Zuk & Stoehr 2002). Due to the specificity of MHC-peptide binding, individual alleles often provide a fitness advantage against some pathogen epitopes while simultaneously incurring a fitness cost.
against others (Gandon 2002; Bernatchez & Landry 2003). Thus, the strength and
targets of selection on the MHC are likely to differ across populations, depending on the
genetic background of the host and the full complement of pathogens at each site. In this
case, our finding that PBR heterozygosity was a fitness advantage across all
populations underscores that the broader peptide binding capabilities inherent to having
two MHC alleles can be more advantageous than having one allele with particular
efficacy against one pathogen epitope (Zuk & Stoehr 2002). Heterozygote advantage
may thus be the general mechanism for enhancing chytridiomycosis resistance in
natural populations. However, we also detected a resistance allele in population SM,
highlighting that individual alleles can be selectively advantageous for specific genetic
backgrounds and environmental regimes. Numerous studies of wildlife populations
demonstrate that genetic disease resistance is a host, strain, and context-dependent
process (Bernatchez & Landry 2003; Apanius et al. 1997), and our results confirm that
lowland leopard frogs have reduced chytridiomycosis susceptibility via multiple
evolutionary mechanisms.

The ability of host populations to evolve *Bd* resistance could be limited due to the
recent timescale of chytridiomycosis emergence. Lowland leopard frogs have suffered
from *Bd*-induced declines for only 20 generations (Bradley et al. 2002), and genetic
theory predicts that the shorter generation time of pathogens should provide them an
advantage in the co-evolutionary arms race (Gandon 2002). However, hosts can benefit
from high standing genetic diversity, because the rate of pathogen evolution is
negatively correlated with host genetic variability (Lipstich et al. 1995; Regoes et al.
2000; Zhan et al. 2002). Empirical studies also demonstrate that relative generation
times of hosts and pathogens do not influence local adaptation (Morgan & Buckling
2006). In fact, when pathogen genetic variability is low, as is the case for *Bd* in the New
World (Morgan et al. 2007), short generations increase the strength of selection for host
resistance and decrease the capacity of pathogens to adapt to an evolving host (Gandon & Michalakis 2002). Chytridiomycosis in \textit{L. yavapaiensis} thus fits a scenario with strong potential for the evolution of heritable disease resistance in host populations. In our study, SM and AC were the only populations where individuals varied in \textit{Bd} survival and the only populations with the PBR allele (Q) showing a signal of recent positive selection. Importantly, these populations also had the lowest proportions of \textit{Bd} mortalities in our field surveys, indicating that our experimental results have biological relevance to \textit{Bd} dynamics in natural populations. These populations are therefore the best candidates for future studies of the evolutionary dynamics of \textit{Bd} resistance in natural populations.

Animal hosts evolve two mechanisms to combat infections: resistance, defined as the ability to limit pathogen burden, and tolerance, the ability to limit the damage caused by a given pathogen burden (Råberg \textit{et al.} 2007). The distinction between tolerance and resistance is critical for understanding the evolutionary consequences of chytridiomycosis disease dynamics, because resistance has a negative effect on pathogen fitness and tolerance does not (Råberg \textit{et al.} 2007). Under our experimental conditions, surviving frogs cleared \textit{Bd} infections, demonstrating disease resistance. However, the same lowland leopard frog populations (AC and SM) show evidence of disease tolerance in the field, where individuals carry high pathogen burdens with no disease signs (Savage \textit{et al.} 2011). These differences underscore the need to consider both experimental infection studies and disease dynamics in wild populations. Controlled experiments allow us to pinpoint the effects of immunogenetic factors in the absence of environmental variation. However, the critical next step will be studies of natural populations incorporating environmental factors that mediate host resistance and tolerance, pathogen virulence, and population MHC variability.
Given the scale of the amphibian biodiversity crisis (Wake & Vredenburg 2008), understanding host-pathogen chytridiomycosis dynamics is essential for amphibian conservation. Our study shows that genetically distinct lowland leopard frog populations experimentally infected with *Bd* have significant differences in survival that correlate with immunogenetic heterozygosity and polymorphism. This is the first experimental demonstration that host genetic variability determines *Bd* infection outcomes under controlled environmental conditions. To date, studies of natural amphibian populations have compared chytridiomycosis susceptibility across sympatric species (Lips et al. 2006; Pounds et al. 2006; Crawford et al. 2010), among intraspecific populations (Rachowicz et al. 2006), and between individuals (Briggs et al. 2005). Our findings emphasize the need for studies that quantify host population genetics and immunogenetics at these same hierarchical levels. For highly susceptible species, captive breeding is one of the few remaining strategies for maintaining natural populations (Mendelson et al. 2006). Species reintroductions with assisted selection could be a viable chytridiomycosis mitigation strategy, but will require a better understanding of amphibian immune responses and immunity genes targeted by natural selection (Woodhams et al. 2011). Although allele Q may be unique to *L. yavapaiensis*, we also detected significant heterozygote advantage, and selection for MHC heterozygosity is a strategy that could be implemented for any *Bd*-susceptible amphibian species. We identify for the first time genetic determinants of chytridiomycosis resistance in natural populations of a declining amphibian, a critical step in developing genetically-informed breeding programs for species recovery.

**Methods**

**Egg Mass Collection and Husbandry.** We collected *L. yavapaiensis* egg masses from five populations in Arizona, USA in March 2008 (Figure S3.1). We detached partial
clutches and shipped them overnight to Cornell University. Frogs were housed in a 12h:12h light:dark regime at 30% humidity (±3%) and 21°C (±1°C) to mimic winter field conditions. Upon hatching, we equally divided larvae from each clutch into eight replicate 16 × 30 × 9 cm plastic cages filled with dechlorinated lab water. We fed larvae 90% spirulina algae and 10% fish flakes ad libitum, replacing water with fresh dechlorinated lab water three times per week. Variable survival resulted in unequal numbers of frogs per replicate, ranging from 1-5 per cage (Table S3.3). We transferred metamorphosed frogs to 16 × 30 × 9 cm plastic cages containing plastic perches and kept moist with a film of dechlorinated lab water. Frogs were fed 3 week old pathogen-free crickets ad libitum. Only metamorphs reaching the stage of complete tail resorption (post-Gosner stage 46) were used in experimental infections.

**Bd and Chytridiomycosis Field Surveys.** We surveyed populations for *Bd* during winter (January–February) of 2007-2010 (Savage *et al.* 2011). We categorized each dead individual testing positive for *Bd* as a chytridiomycosis mortality event. Additionally, we collected frogs with signs of chytridiomycosis (i.e., skin redness, lethargy, and loss of righting ability) for overnight observation, and categorized these as *Bd* mortalities if death occurred within 24h and the individuals were *Bd*-positive. We estimated *Bd* infection and mortality prevalence with 95% Clopper-Pearson binomial confidence intervals (Clopper & Pearson 1934).

**Experimental Bd exposures.** Frogs were infected with *Bd* strain PsTr2004, an Arizona strain isolated from a *Pseudacris triseriata* individual collected from Coconino County, AZ (UTMS: 446716E, 3871380N) in 2003. This locality is geographically distinct from our five populations, minimizing the potential that they were locally adapted to this *Bd* strain. Frogs from each replicate group were split into two cages; one cage was seeded with 1
X $10^5$ PsTr2004 *Bd* zoospores in 1mL of TGhL broth and the other cage was sham-infected with 1mL of TGhL broth only. The film of water/TGhL/Bd or water/TGhL covering the floor of each cage was left undisturbed for 24h, and a new film of dechlorinated lab water was added to each cage daily. Cages were rotated daily to avoid the potential for microclimatic effects. Frogs were weighed immediately prior to infection, and at death or the end of the experiment, whichever came first.

**Infection intensity over time.** To quantify *Bd* infections, we used sterile swabs and followed standard amphibian swabbing protocols (Hyatt *et al.* 2007). *Bd* DNA was extracted using Prepman Ultra, and we used qPCR (Boyle *et al.* 2004) to determine *Bd* infection intensity. Before experiments began, we tested individuals for pre-existing *Bd* infection at all three developmental stages (eggs, larvae and metamorphs). One day prior to experimental *Bd* infections, we assayed all metamorphs for *Bd* infection. Frogs were handled with unused latex gloves to prevent *Bd* transmission. Following *Bd* exposures, we quantified infection intensity for each surviving replicate group on 1, 3, 7, 14, 21, 28 and 35 DPI.

**Statistical Analysis of Survival.** We analyzed survival data using the Cox proportional hazard model (Cox 1972), which assumes an underlying function describing how hazard changes over time and fits effect parameters using Cox’s likelihood. Survival was censored, meaning that individuals may die after the end of the study period. To identify potential predictors, we first performed a univariate analysis for each variable and each interaction among variables. We performed log-rank tests of equality across strata for categorical variables (replicate, clutch, source population, number of cage mates, and MHC heterozygosity), and univariate Cox proportional hazard regression for continuous variables (initial mass, change in mass, and maximum infection intensity). Predictors
with a \( P \)-value of 0.1 or less in the univariate analyses were included as potential effect parameters in the final model. Significant \( P \)-values were determined using sequential Bonferroni correction (Rice 1989). We tested whether infection intensities were significantly different from zero for survivors and non-survivors using Wilcoxon signed-rank tests. Tests were performed in JMP version 9.0 (SAS Institute Inc.).

**Screening of Microsatellite Loci.** We genotyped all \( Bd \)-infected individuals at 14 previously characterized microsatellite loci (Savage & Jaeger 2009). DNA was extracted using a 5% Chelex 100 solution with 0.5 µg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. PCR amplification was performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different labels or non-overlapping size ranges were multiplexed and run on a 3730 Genetic Analyser (Applied Biosystems). Fragment sizes were determined by comparison with a LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems).

**Population Structure.** Genetic structuring among populations was inferred for all \( Bd \)-infected individuals. We used the program Structure version 2.3 (Pritchard et al. 2000b) to identify the most likely number of genetic clusters (\( K \)) present and the proportion of membership in those clusters for each \( Bd \)-infected clutch. This method uses Bayesian assignment techniques to identify clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. Twenty independent runs were performed for each value of \( K \) ranging from 1–12, with a burn-in of one million Markov chain Monte Carlo (MCMC) iterations and a data collection period
of three million MCMC iterations.

**MHC amplification, cloning and sequencing.** We extracted genomic DNA from toe-clips of *Bd*-infected individuals using Qiagen DNeasy kits. We used a degenerate MHC class IIB forward primer (MHC-F; Kiemnec-Tyburczy *et al.* 2010) and an intron-specific reverse primer (B1intron2_R; Hauswaldt *et al.* 2007) to amplify 246 base pairs (bp) of exon 2 and 189 bp of adjacent 3’ flanking intron. This primer pair was designed to amplify alleles from a single locus and ensure that only orthologues were compared, because at least two MHC class IIB loci are expressed in *L. yavapaiensis* (Kiemnec-Tyburczy *et al.* 2010). Subsequent analyses included the exon 2 coding region only.

Amplifications were performed for 35 cycles at 95°C for 50 s; 60°C for 45 s; and 72°C for 1 min. We cloned PCR products into the Promega pGEM® T vector and transformed recombinant DNA into Invitrogen TOP-10 *E. coli*. Cells were grown on Luria agar plates for 18-22 hours at 37°C. We used blue/white screening to choose 8-32 clones from each transformation and amplified them using M13 primers with standard reaction conditions. PCR products were purified using an alkaline phosphatase-exonuclease reaction and sequenced on an ABI 3730 Sequencer using Big Dye v3.1 chemistry. We discarded MHC sequences recovered singly with ≤ 2 nucleotide polymorphisms to other sequences, attributing these small differences to PCR or cloning error. After discarding these sequences, no more than two alleles were recovered from any individual. We used translated amino acid queries in GenBank to confirm MHC class IIB homology and aligned sequences in Sequencher v. 4.10 (Gene Codes Corporation).

**Genealogy reconstruction.** We tested the alignment of MHC sequences for recombination using Genetic Algorithm for Recombination Detection (Kosakovsky Pond
et al. 2006), and detected no recombination, thus we performed an unrooted maximum likelihood (ML) analysis. Model parameters were determined using Akaike and Bayesian information criteria in jModeltest version 0.1.1 (Posada 2008). The best-fit model (HKY85) was used in a ML analysis in PAUP* (Sinauer Associates) using the heuristic search option, TBR branch swapping, MulTrees option in effect, and a single neighbor-joining tree as a starting topology. Node support was estimated from 1000 bootstrap replicates.

**Tests of selection.** We ran tests of selection using the HyPhy software package (Kosakovsky Pond et al. 2005) with the ML genealogy as our input tree. We used the PARRIS method to test for positive selection across the alignment, and the Evolutionary Fingerprinting method to infer positive selection rate classes and selection intensity. We tested for residue-specific selection in PBR lineages using three maximum likelihood methods: Single Likelihood Ancestral Counting (SLAC), Fixed Effects Likelihood (FEL), and Random Effects Likelihood (REL; Kosakovsky Pond et al. 2005).

**Analysis of risk factors.** We calculated Relative Risk (RR) using equation (1):

\[
RR = \frac{(p^+ \times c^-)}{(p^- \times c^+)} ,
\]

where \( p^+ \) is the frequency of frogs with allele \( x \) that died, \( p^- \) is the frequency of frogs with allele \( x \) that did not die, \( c^- \) is the frequency of frogs without allele \( x \) that died, and \( c^+ \) is the frequency of frogs without allele \( x \) that did not die. Significance of each RR value was assessed using Fisher's exact test and sequential Bonferroni correction (Rice 1989).
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CHAPTER 4:

IMMUNOGENETIC ADAPTATION TO AN EMERGENT INFECTIOUS DISEASE IN
NATURAL POPULATIONS OF A DECLINING AMPHIBIAN

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Abstract

For the last three decades, amphibians have been declining worldwide due to the emerging fungal disease chytridiomycosis, but we are just beginning to understand how immunogenetic variability contributes to disease susceptibility. We recently showed that lineages of an expressed Major Histocompatibility Complex (MHC) class II locus involved in acquired immunity associate with chytridiomycosis resistance and susceptibility among frog populations and individuals in controlled laboratory challenge assays. Here, we extended these findings to a survey of natural populations that vary in their exposure and response to the pathogen *Batrachochytrium dendrobatidis* (*Bd*). We found that MHC allelic lineages associated with disease resistance in the field show a molecular signal of positive selection, while those associated with susceptibility do not, confirming that heritable disease resistance may be rapidly evolving in the wild. We confirmed that natural selection has shaped MHC variability by comparing allele frequencies of MHC exons to neutral loci. In a population with complete *Bd* resistance in nature, we detected a significant signal of directional selection for the same chytridiomycosis resistance allele (allele Q) that showed a significant association to survival in our lab experiments. Our findings indicate that strong selective pressure for
disease resistance can drive rapid immunogenetic adaptation in natural populations, despite differences in environment, demography, and the many other factors that differ among natural populations. Our field-based survey of immunogenetic variation confirms that natural amphibian populations have the evolutionary potential to adapt to chytridiomycosis.

**Introduction**

Emerging infectious disease in natural populations is among the five leading threats to biodiversity, and mitigating its effects is a top conservation priority (Balmford et al. 2005). Amphibians are early indicators of environmental change (Blaustein & Wake 1990; Welsh & Ollivier 1998), thus the amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), raises particular concern for biodiversity and human health (Daszak et al. 2000). Chytridiomycosis has resulted in the decline or extinction of hundreds of species worldwide (Berger et al. 1998; Skerratt et al. 2007). Amphibian species demonstrate a wide range of responses to chytridiomycosis (Stuart et al. 2006) that are largely driven by environmental and ecological factors (Vredenburg et al. 2010; Becker & Zamudio 2011; Rohr et al. 2011; Searle et al. 2011). Host immunological responses also contribute to *Bd* susceptibility (Woodhams et al. 2007; Richmond et al. 2009; May et al. 2011; Rollins-Smoth et al. 2010; Savage & Zamudio 2011), but it has been difficult to identify differences in resistance among species or natural populations because of the confounding effects of environmental control of pathogen dynamics (Piotrowski et al. 2004; Rohr et al. 2008) and demographic factors contributing to disease. Thus, the potential evolution of hosts in response to this emergent disease remains underexplored in natural populations.

Amphibian immune systems are structurally and functionally similar to other vertebrate animals in possessing innate and acquired immune pathways with cellular
and humoral pathogen destruction mechanisms (Du Pasquier et al. 1989). A unique feature of the amphibian immune system is the production of diverse antimicrobial skin peptides, some of which are correlated with higher Bd resistance (Woodhams et al. 2007). Frog antibodies capable of binding to Bd have also been identified in infected individuals (Ramsey et al. 2010), suggesting humoral immune defenses are activated in response to Bd infection. In addition to these mechanisms, a likely immune system component contributing to host Bd responses is the Major Histocompatibility Complex (MHC), a family of immune-related genes that are conserved across vertebrate animals (Ohta et al. 2000). Class I and II MHC molecules must bind pathogen molecules on their peptide-binding regions (PBRs) and present them to T-cells to initiate an acquired (as opposed to innate) immune response (Jones et al. 2006). This central role in initiating and regulating immune responses creates strong selection on MHC loci for numerous polymorphisms and gene copies, thereby maximizing the array of pathogens that can be recognized (Bernatchez & Landry 2003; Shiina et al. 2004). Class II MHC genes are expressed on immune surveillance cells in amphibian skin (Du Pasquier et al. 1989; Carillo-Farga et al. 1990) and typically recognize bacterial and fungal pathogens, whereas class I molecules are involved primarily in viral immunity and self-discrimination (Bevan 1987). Class II loci are therefore ideal targets for immunogenetic studies of chytridiomycosis, a fungal disease that is restricted to amphibian epidermal cells (Berger et al. 1998).

Natural wildlife populations commonly show correlations between MHC polymorphism and disease susceptibility (Bernatchez & Landry 2003). Four non-exclusive evolutionary mechanisms may explain the response of MHC allele distributions after pathogen-imposed selection in populations. First, overdominance may arise if individuals that are heterozygous at MHC loci are able to bind a wider inventory of antigens (Hughes & Nei 1989; Hughes & Nei 1992), resulting in higher fitness compared
to homozygotes (Doherty & Zinkernagel 1975; Thursz et al. 1997; Buchbinder et al. 1999). Second, directional selection may occur if a specific allele (or lineage of alleles) increases in frequency over successive generations because it confers resistance to a common pathogen imposing strong selective pressure (Teacher et al. 2009; Vassilakos et al. 2009). Third, frequency-dependent selection may occur when rare MHC alleles have a selective advantage because pathogens are selected to be adapted to the most common host genotype. Once those rare MHC alleles increase in frequency as a consequence of their advantage against common alleles (Ladle 1992, Ebert & Hamilton 1996), pathogens in turn adapt to them, resulting in a balanced polymorphism of numerous alleles (Clarke & Kirby 1966; Takahata & Nei 1990; Meyer & Thomson 2001). Finally, diversifying selection may take place if spatially heterogeneous selection from multiple pathogens or pathogen strains selects for numerous resistance-conferring alleles within populations (Hill 1998), leading to balanced MHC polymorphism (Hedrick 2002; Bernatchez & Landry 2003) that is indistinguishable from frequency-dependent selection. Some or all of these mechanisms have likely shaped MHC diversity over the history of natural wildlife populations; thus, teasing apart the specific immunogenetic consequences of Bd-imposed selection will require multiple lines of evidence and the ability to distinguish historical versus recent selective events.

In amphibians, the MHC has only been extensively characterized in two model frog species, Silurana (Xenopus) laevis (Kaufman et al. 1985; Flajnik & Du Pasquier 1990) and S. tropicalis (Ohta et al. 2006). Silurana has the ancestral tetrapod MHC gene organization (Nonaka et al. 1999), making it a good model for higher-level comparative studies of MHC evolution (Flajnik et al. 1990). However, Silurana diverged relatively early in the anuran phylogeny (Frost et al. 2006) making it a poor model for MHC evolution in the remaining 6000+ frog species. Recent experimental studies in Silurana find that under some conditions, Bd infection activates innate but not acquired immune
defenses (Ribas et al. 2009) or minimal immune responses of any kind (Rosenblum et al. 2009), while under other conditions, acquired immunity plays a role in host responses (Ramsey et al. 2010). However, Silurana appears to be resistant to chytriidiomycosis in the wild, never showing clinical signs of disease despite *Bd* infections (Weldon et al. 2004), calling into question the applicability of these studies to amphibian species highly susceptible to chytriidiomycosis. MHC genes have been examined in few natural populations of non-model amphibians (Sammut et al. 199; Bos & DeWoody 2005; Hauswaldt et al. 2007; Teacher et al. 2009; Kiemnec-Tyburczy et al. 2010). Only one study to date explores MHC dynamics in relation to chytriidiomycosis: in larval populations of the natterjack toad (*Bufo calamita*), MHC class II genotype frequencies varied in a pattern consistent with directional selection in response to pathogen prevalence among populations (May et al. 2011). Combined, these studies suggest a role for MHC genes in chytriidiomycosis dynamics, but further studies of naturally susceptible species are necessary to elucidate the precise mechanisms underlying the evolution of immunogenetic resistance to *Bd*.

The lowland leopard frog (*Lithobates yavapaiensis*) is a North American amphibian that has been declining from seasonal chytriidiomycosis outbreaks for at least twenty years (Bradley et al. 2002; Savage et al. 2011a). We recently reported on experimental *Bd* infections of lab-reared lowland leopard frogs originating from five populations, that showed specific MHC genotypes associated with survival, both within and among populations (Savage & Zamudio 2011). In particular, we found that PBR heterozygotes and individuals bearing PBR allele Q had significantly higher probabilities of surviving *Bd* infection (Savage & Zamudio 2011). Here, we test the generality of those findings in natural populations; we characterize the same PBR region of an expressed MHC class II gene (Kiemnec-Tyburczy et al. 2010) in field-sampled frogs from eight lowland leopard frog populations currently infected with *Bd* and interpret genetic
variation at this putative functional locus in light of multi-year field estimates of population and individual *Bd* susceptibilities. We also characterize two neutral genetic markers from these same populations to compare neutral and immunogenetic genotypes in the context of disease dynamics and identify significant signals of natural selection in response to chytridiomycosis. Our efforts to identify host immunogenetic factors that contribute to *Bd* susceptibility in natural populations complement our lab-based association studies by elucidating the mechanisms of evolutionary response to disease across a variable ecological and environmental landscape.

Results

*Intra- and Interpopulation Variation in Field Bd Disease Dynamics*

Lowland leopard frog populations are genetically and geographically distinct (Table S4.1; Table S4.2) and respond differently to *Bd* infection (Figure 4.1). In three populations (HR, SM and SS), *Bd* infected frogs persist without mortality, while in five populations (AC, CIC, MR, TV, and WC), a proportion of individuals develop chytridiomycosis and die (Figure 4.1A), but that proportion varies significantly across populations, with mortality being highest in population TV and lowest in AC. In a previous laboratory study, we performed *Bd* challenge assays on frogs collected from AC, CIC, MR, SM and WC (Savage & Zamudio 2011; hereafter referred to as our experimental infection study), and we found a strong association with *Bd* resistance and two immunogenetic factors: class II MHC heterozygosity, and the presence of class II allele Q.

In addition to differences in *Bd* infection prevalence, infection intensity (measured as the logarithm of the number of genome equivalents recovered per swab) also varies across natural populations (Figure 4.1B). Surprisingly, some populations with no mortality have higher mean infection intensities than other populations with mortality.
(e.g., HR versus WC; Figure 4.1B), suggesting that infection intensities have functionally different consequences in different locations. However, within populations, frogs that died had significantly higher mean $Bd$ infection intensities than frogs that survived (two-tailed paired Student's t-test, $P=0.046$; Figure 4.1C), confirming that an inability to limit pathogen burden is the proximate cause of $Bd$-associated mortality within populations that show susceptibility to $Bd$. At present, we are incapable of clearly distinguishing between $Bd$ resistance (limiting pathogen burden) and tolerance (limiting the damage incurred by a given pathogen burden; Råberg et al. 2007), and how the two mechanisms differ within and among populations, thus for simplicity we hereafter refer to any process of surviving $Bd$ infection as resistance, with the knowledge that tolerance is involved in at least some populations.
Figure 4.1: *Batrachochytrium dendrobatidis* (*Bd*) infection and chytridiomycosis mortality vary among *Lithobates yavapaiensis* populations. (A) Prevalence of *Bd* infection (proportion of frogs testing positive for *Bd*; open symbols) and mortality (proportion of frogs that died; closed symbols) in populations with and without chytridiomycosis mortality. AC = Aravaipa Canyon; CIC = Cienega Creek; HR = Hassayampa River; MR = Muleshoe Ranch; SM = Santa Maria River; SS = Seven Springs; TV = Tanque Verde Canyon; WC = Willow Creek; error bars indicate 95% binomial confidence intervals. (B) Logarithm of the mean *Bd* infection intensity (genome equivalents +SD) among individuals from populations with (closed symbols) and without (open symbols) chytridiomycosis mortality. (C) Logarithm of the mean *Bd* infection intensity (genome equivalents +SD) among surviving individuals (open symbols) and dying individuals (filled symbols) in populations with chytridiomycosis mortality.

**MHC Alleles and Lineages Associate with *Bd* Resistance**

We cloned and sequenced MHC class II PBR alleles from a single expressed locus (Kiemnec-Tyburczy et al. 2010) for 128 frogs from eight populations in Arizona, USA (Figure S3.1). We identified 84 unique PBR alleles, and we reconstructed a Bayesian genealogy to examine their distribution among populations with and without chytridiomycosis mortality (Figure 4.2). Three PBR alleles were recovered at high frequency (alleles A, N and Q); the same three alleles that were recovered in our earlier experimental *Bd* infection of lab-reared frogs (Savage & Zamudio 2011). Sixty-six of the 84 alleles were singleton private alleles (SPA), meaning the sequence was recovered once among all individuals sequenced from eight populations. An additional seven sequences were private alleles that were recovered more than once, but only from a single population. Due to the high proportion of singleton alleles, in addition to analyzing genetic association of the common alleles (A, N, Q) with *Bd* susceptibility, we also tested
for associations of clades with ≥99% support and ≥5 alleles recovered from dead
individuals (Clades R1, R2 and S1, Figure 4.2), as groups of closely related alleles may
bind the same pathogen epitopes and provide the same level of protective immunity
(MacDonald et al. 2000).

To explore associations between PBR genotypes and Bd dynamics within
populations, we examined the distribution of the three high-frequency alleles (A, N, and
Q), the three well-supported clades (R1, R2 and S1), and PBR heterozygotes versus
homozygotes among individual frogs found dead or alive in the field (Table 4.1). PBR
allele A and clade S1 were both significantly associated with disease susceptibility;
individuals with these alleles had more than a three-fold increased risk of death. Notably,
allele A was also significantly associated with mortality in experimentally infected
lowland leopard frogs (Savage & Zamudio 2011). In contrast, PBR allele Q and clade R1
were significantly associated with disease resistance; individuals with these sequences
had a three- to four-fold reduced risk of death. Allele Q was the only allele that also
showed a significant association with survival in experimentally infected lowland leopard
frogs (Savage & Zamudio 2011). Although clade R2 did not show a significant
association with survival due to its small sample size, this allele was only recovered from
surviving individuals from a Bd-resistant population (SS). Surprisingly, PBR
heterozygotes showed no reduced risk of mortality in the field, despite a strong survival
advantage of heterozygosity in experimentally infected frogs (Savage & Zamudio 2011).
Figure 4.2: MHC class II peptide-binding region (PBR) lineages experience positive selection and associate with chytridiomycosis mortality across frog populations and individuals. Maximum likelihood genealogy of 84 PBR sequences. Alleles from populations with mortality are in black and those from populations without mortality are in white, with circle size proportional to frequency. One high frequency allele associates with populations that do not experience chytridiomycosis mortality (I), while another associates with populations that do (II). Significantly elevated nonsynonymous substitution rates occur along three terminal branches leading to MHC alleles from four populations. Alleles from individuals in populations without mortality are in white, from dying individuals in populations with mortality are in black, and from surviving individuals in populations with mortality are in gray. The codon positions and amino acid substitutions occurring along lineages under positive selection are in boxes. Posterior Probabilities (PP) >70% are indicated. Population name abbreviations follow Figure 4.1. Dashed lines indicate spacing for presentation purposes and are not branches.

Table 4.1: MHC risk factors associated with *Bd* mortality.

<table>
<thead>
<tr>
<th>MHC Risk Factor</th>
<th>Relative Risk*</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygote</td>
<td>0.68</td>
<td>0.22</td>
<td>0.35 – 1.30</td>
</tr>
<tr>
<td>Clade R1</td>
<td>0.40</td>
<td>&lt;0.0001</td>
<td>0.23 – 0.68</td>
</tr>
<tr>
<td>Clade R2</td>
<td>0.00</td>
<td>0.12</td>
<td>0.00 – 1.41</td>
</tr>
<tr>
<td>Clade S1</td>
<td>3.64</td>
<td>&lt;0.0001</td>
<td>2.15 – 6.37</td>
</tr>
<tr>
<td>Allele A</td>
<td>3.20</td>
<td>&lt;0.0001</td>
<td>2.10 – 4.79</td>
</tr>
<tr>
<td>Allele N</td>
<td>0.51</td>
<td>0.091</td>
<td>0.20 – 1.14</td>
</tr>
<tr>
<td>Allele Q</td>
<td>0.23</td>
<td>0.008</td>
<td>0.04 – 0.86</td>
</tr>
</tbody>
</table>

*Significant values (after Bonferroni correction) are shown in bold for reduced risk of mortality, and bold italics for increased risk of mortality.*
**Codon 46 Is Positively Selected in Lineages Associated with Resistance**

To distinguish selection at MHC loci from neutral evolution, we examined nucleotide substitution rate ratios within the 83 codons of our PBR alleles. A substitution rate ratio is the ratio of nonsynonymous nucleotide substitutions per nonsynonymous sites (dN) to synonymous substitutions per synonymous sites (dS), or $\omega$, for each codon position in a sequence alignment. Values of $\omega$ that are significantly greater than 1 indicate positive or diversifying selection, whereas values of $\omega$ that are significantly less than 1 indicate negative or purifying selection. Evolutionary models that included positive selection ($\omega > 1$ for a proportion of codons) fit the alignment of PBR sequences significantly better than models excluding positive selection (Evolutionary Fingerprinting, log likelihood = -1951.54, $P < 0.001$). The best-fit model included three nucleotide substitution rate classes, and estimated that 65% of codons experienced negative selection ($\omega = 0.78$), 30% of codons experienced moderate positive selection ($\omega = 1.28$), and 5% of codons experienced strong positive selection ($\omega = 1.95$). Using the most conservative codon-specific test of selection, Single Likelihood Ancestral Counting (SLAC), we detected a significant signature of positive selection acting on codon 46 of the PBR alignment ($P = 0.05$, Normalized dN–dS = 4.08). Positive selection was detected at codon 46 along the branches leading to clades R1 and R2, but not clade S1 (Figure 4.2, highlighted branches). This residue is located at one of the 14 codon positions that determine peptide binding in the human MHC class II PBR (Tong et al. 2006), corroborating its function in binding pathogen peptides to initiate amphibian acquired immune responses. Further, this was the same residue where a significant signal of positive selection was detected in our experimental infection study (Savage & Zamudio 2011). This result corroborates the finding that codon 46 may be particularly important in determining *Bd* peptide binding, such that a single nonsynonymous
substitution has resulted in a chytridiomycosis resistance allele.

**Neutral and PBR Loci Show Discordant Patterns of Population Differentiation**

To disentangle the genetic signatures of selection and demography, we compared MHC class II PBR genotype frequencies to genotype frequencies of two putatively neutral markers: (1) the adjacent MHC intron, and (2) 14 microsatellite loci (Figure 4.3). Concordant patterns among neutral and MHC loci would indicate that demography dictates population differentiation. In contrast, directional or balancing selection for the same MHC allele(s) across populations should produce lower differentiation at MHC compared to neutral loci, whereas if selection on the MHC is distinct in each locality, populations should be more differentiated at MHC compared to neutral loci. For all eight populations, indices of population genetic differentiation ($F_{ST}$) estimated from MHC versus neutral markers are incongruent, demonstrating that selection and not demography has shaped MHC differentiation among populations. In some population comparisons, neutral markers show higher differentiation compared to MHC (Figure 4.3; values below the dashed line), a pattern expected if balancing selection is homogenizing MHC allele frequencies and reducing between-population differentiation. In the other population comparisons, MHC differentiation is higher than neutral markers (Figure 4.3; values above the dashed line), consistent with directional selection for different MHC alleles among those population pairs.

The MHC intron is in tight linkage with the PBR exon under selection, whereas microsatellite loci are distributed throughout the genome, thus only very recent demographic processes would differentiate results from these two loci. We detected the same pattern of differentiation using microsatellites and intronic sequences; however, the incongruence between PBR and neutral pairwise $F_{ST}$ measures was higher when comparing PBR genotypes to microsatellite genotypes. HR was the only population with
all pairwise population comparisons falling above the dashed line (Figure 4.3B), indicating directional selection for a single PBR allele, while in all other populations, some pairwise comparisons indicated higher MHC differentiation and others indicated lower MHC differentiation. Combined, indices of population differentiation demonstrate that selection is shaping PBR evolution, but the mode(s) of selection are distinct in each population, perhaps reflective of the different pathogen epidemiologies across localities (Figure 4.1B).

Figure 4.3: MHC class II PBR alleles show signatures of selection in *Lithobates yavapaiensis* populations. (A) Pairwise population differentiation indices ($F_{ST}$) calculated from MHC class II PBR exon and MHC class II intron genotypes. (B) Pairwise population differentiation indices ($F_{ST}$) calculated from MHC class II PBR exon and 14-locus microsatellites.
microsatellite genotypes. The dashed line represents perfect concordance among $F_{ST}$ values measured from both genetic marker types; values above the line indicate higher differentiation at PBR genotypes, and values below the line indicate higher differentiation at non-coding genotypes. Filled symbols represent populations with mortality and open symbols represent populations without mortality.

**Neutral and PBR Loci Detect Population Histories of Directional Selection and Demographic Expansion**

We inferred the mode of selection acting within each population by examining neutral and MHC genotypes. Genetic diversity was significantly higher for PBR exons compared to introns when measured either as nucleotide diversity ($\pi$; two-tailed paired Student’s t-test, $P=0.014$) or the number of segregating sites ($\theta$; two-tailed paired Student’s t-test, $P=0.0097$; Table 4.2). We conducted Ewens-Watterson, Tajima’s $D$, and Fu’s $F_S$ tests of selective neutrality on MHC class II PBR exon alleles to infer signatures of directional or balancing selection, and also performed these tests on MHC intron and microsatellite alleles (where possible) as a control for the effects of demography (Nielsen et al. 2009). For the PBR exon, at least one of the three tests produced a significant signal of directional selection for populations AC, CIC, HR, SM, and TV; in contrast, we detected no signal of directional selection for populations MR, SS and WC, and no significant signal of diversifying selection in any population (Table 4.2, Figure 4.4).
Table 4.2: Population genetic variability and signatures of selection.

<table>
<thead>
<tr>
<th>Pop.</th>
<th>Genetic region</th>
<th>E-W test P-value</th>
<th>Tajima’s D (P-value)</th>
<th>Fu’s F&lt;sub&gt;S&lt;/sub&gt; (P-value)</th>
<th>(\pi)</th>
<th>(\theta)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Populations with mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIC† (N=24)</td>
<td>MHC exon</td>
<td>1.00*</td>
<td>-2.1 (0.004)</td>
<td>-1.8 (0.25)</td>
<td>2.97</td>
<td>7.89</td>
</tr>
<tr>
<td></td>
<td>MHC intron</td>
<td>0.99</td>
<td>-1.5 (0.06)</td>
<td>-3.7 (0.04)</td>
<td>1.70</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>Microsatellites</td>
<td>0.73</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MR† (N=15)</td>
<td>MHC exon</td>
<td>0.65</td>
<td>-0.43 (0.40)</td>
<td>1.27 (0.74)</td>
<td>18.5</td>
<td>9.76</td>
</tr>
<tr>
<td></td>
<td>MHC intron</td>
<td>0.85</td>
<td>-0.21 (0.47)</td>
<td>-1.2 (0.24)</td>
<td>2.21</td>
<td>2.39</td>
</tr>
<tr>
<td></td>
<td>Microsatellites</td>
<td>0.92</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>TV (N=15)</td>
<td>MHC exon</td>
<td>0.99</td>
<td>-1.9 (0.01)</td>
<td>0.66 (0.67)</td>
<td>2.72</td>
<td>5.96</td>
</tr>
<tr>
<td></td>
<td>MHC intron</td>
<td>0.99</td>
<td>-1.9 (0.01)</td>
<td>-4.3 (0.004)</td>
<td>1.52</td>
<td>3.48</td>
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<tr>
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<td>Microsatellites</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WC† (N=8)</td>
<td>MHC exon</td>
<td>0.84</td>
<td>1.36 (0.93)</td>
<td>2.41 (0.86)</td>
<td>10.4</td>
<td>7.84</td>
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<tr>
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<td>MHC intron</td>
<td>0.92</td>
<td>1.00</td>
<td>0.80 (0.81)</td>
<td>3.79</td>
<td>3.14</td>
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<tr>
<td></td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AC‡ (N=20)</td>
<td>MHC exon</td>
<td>0.99</td>
<td>-1.4 (0.063)</td>
<td>-1.0 (0.37)</td>
<td>20.9</td>
<td>27.5</td>
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<tr>
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<td>MHC intron</td>
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<td>-0.67 (0.31)</td>
<td>-7.6 (0.001)</td>
<td>2.63</td>
<td>3.33</td>
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<tr>
<td></td>
<td>Microsatellites</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Populations without mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (N=19)</td>
<td>MHC exon</td>
<td>1.00</td>
<td>-2.1 (0.004)</td>
<td>-3.4 (0.09)</td>
<td>3.46</td>
<td>8.81</td>
</tr>
<tr>
<td></td>
<td>MHC intron</td>
<td>0.99</td>
<td>-1.6 (0.04)</td>
<td>-3.3 (0.05)</td>
<td>2.10</td>
<td>4.05</td>
</tr>
<tr>
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<td>Microsatellites</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SM‡ (N=14)</td>
<td>MHC exon</td>
<td>1.00</td>
<td>-1.27 (0.10)</td>
<td>0.26 (0.55)</td>
<td>8.69</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>MHC intron</td>
<td>0.97</td>
<td>-0.024 (0.5)</td>
<td>-1.4 (0.22)</td>
<td>2.55</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>Microsatellites</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SS (N=11)</td>
<td>MHC exon</td>
<td>0.76</td>
<td>-0.20 (0.49)</td>
<td>0.78 (0.66)</td>
<td>17.3</td>
<td>14.5</td>
</tr>
<tr>
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<td>MHC intron</td>
<td>0.50</td>
<td>0.48 (0.75)</td>
<td>-1.1 (0.31)</td>
<td>5.27</td>
<td>4.66</td>
</tr>
<tr>
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<td>Microsatellites</td>
<td>0.92</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Bold values indicate a significant signature of directional selection (\(P > 0.95\) for Ewens-Watterson tests and \(P < 0.05\) for Tajima’s \(D\) and Fu’s \(F_S\) tests), and italicized values indicate discordance among test results for different genetic markers.

† Experimentally \(Bd\)-infected population with 100% mortality in the lab

‡ Experimentally \(Bd\)-infected population with surviving individuals in the lab
The three populations without *Bd* mortality showed distinct selective and demographic patterns. A significant signal of directional selection in population HR was congruent among the MHC exon and intron for Ewens-Watterson and Tajima’s *D* tests, but neither locus showed a significant pattern for Fu’s *F*<sub>S</sub>, demonstrating that selection and not demographic expansion produced the observed patterns. In contrast, population SM had congruent significant tests from PBR exon, intron, and microsatellite alleles, indicating that demographic processes dominate. Finally, population SS showed no significant patterns from any loci. However, genealogical analysis found SS to be the only population with a unique clade of alleles, with a strong signal of positive selection acting on that allelic lineage (Figure 4.2), suggesting the clade may have recently diversified, possibly due to enhanced *Bd* resistance conferred by those alleles.

The five populations with *Bd* mortality did not show evidence of selection acting on PBR alleles; four of them showed significant patterns of historical demographic events and one showed no significant deviations from neutrality. Populations AC, CIC and TV had significant signals of directional selection from PBR alleles, but Fu’s *F*<sub>S</sub> tests, the most powerful tests for detecting population growth (Ramos-Onsins & Rozas 2002), were only significant for the PBR intron, indicating that the high frequency alleles in these populations reflect low MHC diversity following population expansion after initial *Bd*-induced bottlenecks, rather than directional selection. Population WC only had a significant Ewens-Watterson test for the MHC intron, also indicating demographic rather than selective processes. Consistent with demographic rather than selective explanations for patterns of MHC diversity, the high frequency alleles in these four populations are allele A, which associates with mortality, and allele N, which shows no *Bd*-associated pattern (Table 4.1). Finally, population MR showed no selective or demographic patterns from any genetic loci, likely due to the constant source of juvenile recruits from a nearby thermal spring that is environmentally sheltered from *Bd* and
experiences no selection for resistance (Savage et al. 2011a).

In summary, two of the populations without *Bd* mortality (HR and SS) show evidence of selection for PBR alleles associated with resistance, while in populations with *Bd* mortality, demographic processes (AC, CIC, TV, and WC) or neutral evolution (MR) explain neutral and MHC genetic patterns.

**Figure 4.4:** Class II PBR allele distributions of within populations. Filled and open bars represent alleles recovered from dead and live frogs, respectively. Grey bars represent individuals sampled in summer with unknown disease outcomes. Shaded bars denote the allele (Q) associated with *Bd* survival in controlled experimental infections (Savage & Zamudio 2011). Singleton private alleles (S.P.A.) are grouped together as a total proportion of the alleles recovered from each population.
Discussion

Lowland leopard frogs have been declining in recent decades due to chytridiomycosis outbreaks, habitat loss, and invasive species (Witte et al. 2008; Bradley et al. 2002). The seasonal selective pressure imposed by Bd (Savage et al. 2011a) means that populations either must adapt, persist, or they become extirpated. Our results indicate that some populations are adapting to Bd via increased immunogenetic resistance, while other populations lack resistance-conferring MHC alleles and face ongoing chytridiomycosis declines. Our genealogical analyses revealed that MHC allelic lineages are not evenly distributed across populations, with alleles recovered from Bd resistant populations clustered in clades R1 and R2 and alleles recovered from Bd susceptible populations clustered in clade S1 (Figure 4.2). Additionally, positive selection acting on a pathogen-binding residue of the PBR only occurred along lineages leading to resistance-associated clades and alleles (Figure 4.2; Table 4.1). Thus, at the time of initial chytridiomycosis outbreaks, populations with standing MHC variation that included resistance alleles, or with large enough population sizes to generate novel Bd-resistance alleles for natural selection to act upon, have evolved partial or complete Bd resistance. In contrast, populations without standing immunogenetic variation for Bd resistance that were decimated by initial chytridiomycosis outbreaks now have small populations sizes, limited genetic diversity for selection to act upon, and struggle to persist in the face of repeated bouts of Bd mortality (Savage et al. 2011a).

Demographic processes can produce population genetic patterns that mimic the signatures of natural selection (Nielson et al. 2009). For example, population bottlenecks and population expansions can produce an excess or deficiency of heterozygotes, respectively (Watterson et al. 1986). Thus, our comparison of MHC genotypes to neutral
genetic loci further elucidates the evolutionary mechanisms enabling or inhibiting population adaptation to emergent diseases. Because the PBR exon and intron sequences are adjacent and therefore in tight linkage disequilibrium, a shift in the frequency of a PBR exon should produce the equivalent shift in intron allele frequencies. Differences between PBR exons and introns therefore reflect very recent and/or strong selection, whereas differences between PBR alleles and multi-locus microsatellites can reflect selective shifts over longer periods of time (Barton 1986; Charlesworth 2009).

(Patterns of population differentiation reflect this difference; pairwise differentiation measured from PBR exons and introns produce similar values, whereas PBR exons and microsatellite markers produce discordant measures of differentiation (Figure 4.3). However, this pattern is not consistent; some pairwise $F_{ST}$ comparisons reflect higher PBR differentiation and others reflect higher neutral differentiation, underscoring that some populations show similar PBR allele frequency distributions, producing excessively low PBR differentiation measures, and others do not, resulting in disproportionately high levels of PBR differentiation (Figure 4.4). A notable exception to this pattern is population HR, which shows consistently high measures of PBR differentiation compared to both PBR intron and microsatellites, indicative of recent directional selection for a PBR allele in this, but no other, population.

Both directional (Harf & Sommer 2005; Cutrera & Lacey 2007) and diversifying (Paterson et al. 1998; Bonneau et al. 2005) selection on MHC alleles are commonly observed evolutionary responses to emergent pathogens in natural populations. Given that Bd currently imposes strong selective pressure in susceptible lowland leopard frog populations, we might expect directional selection for one or a subset of MHC alleles to predominate in our system. Indeed, significant Ewens-Watterson and Tajima’s $D$ tests, high PBR differentiation compared to neutral markers, and an allele frequency of 0.63 for allele Q indicate that HR, a population with Bd infection but no mortality in the last five
years (Figure 4.1; Savage et al. 2011a), experiences directional selection for allele Q, the Bd resistance allele previously identified in the laboratory (Savage & Zamudio 2011). However, in SS and SM, the other two populations that harbor Bd but show no mortality, the mechanisms underlying resistance are less clear. Population genetics at neutral and PBR loci in SM are consistent with demographic expansion alone, and PBR alleles recovered from this population show no associations with resistance, thus environmental factors, pathogen variation, or other uncharacterized genetic loci may be predominantly responsible for the observed lack of disease. In population SS, the absence of a population genetic signal of selection is likely due to the recent emergence of a group of closely related resistance alleles (Figure 4.2, clade R2); if this population continues expanding in future generations, further sampling may reveal a significant pattern of directional or diversifying selection for one or all of these alleles. Finally, the lack of any signals of selection acting on PBR genotypes in the five populations with Bd mortality is consistent with their ongoing Bd susceptibility; if any of these populations develop Bd resistance in future generations, we would expect a concomitant increase in the frequency of one or a few PBR alleles. Indeed, continued population monitoring in future generations may provide direct evidence for the benefits of particular PBR alleles if the expected correlated changes in allele frequency and disease susceptibility occur. Among our sampled populations, AC has the highest likelihood of evolving future disease resistance, as this population currently harbors allele Q at low frequency.

Collectively, our analyses show that selection for chytridiomycosis resistance has acted on a class II MHC locus in the declining lowland leopard frog. In population HR, Bd-induced selection has generated a high frequency of allele Q, consistent with adaptation arising from standing genetic variation (Feder et al. 2003; Colosimo et al. 2005). Notably, allele Q occurred in all but one of the surviving individuals in experimental Bd infections (Savage & Zamudio 2011). Thus, allele Q has a confirmed
role in providing enhanced \( Bd \) resistance, and is likely responsible for the absence of \( Bd \) mortality in population HR. However, allele Q also occurred at low frequency in four other populations (AC, MR, SM and WC) with no signal of directional selection, suggesting that selection for allele Q may not be strong enough to overwhelm neutral and demographic processes in some of the smaller lowland leopard frog populations that continue to decline from disease. Alternately, the benefit of allele Q may vary across populations due to eco-immunological differences in the host or the pathogen under distinct environmental regimes (Rollins-Smith \textit{et al}. 2011).

In addition to selection acting on standing genetic variants, novel PBR alleles providing \( Bd \) resistance equal to or better than allele Q may have arisen in some populations. We found evidence of this in population SS, which has a low frequency of allele Q, no observed \( Bd \) mortality, and a clade of private alleles (clade R2) with a significant signal of positive selection acting on the same amino acid residue detected for allele Q. Although these sequences were not recovered at high enough frequency to measure a significantly reduced relative risk (Table 4.1), the observed pattern suggests that adaptation to \( Bd \) may have occurred rapidly in population SS from \textit{de novo} genetic mutations, a less frequently documented phenomenon (Linnen \textit{et al}. 2009).

Global declines caused by chytridiomycosis have had catastrophic consequences for amphibian diversity (Skerratt \textit{et al}. 2007) and \( Bd \) continues to spread to new regions and hosts (Cheng \textit{et al}. 2011; Savage \textit{et al}. 2011b). Our study highlights the importance of examining fine-scale demographic, epidemiological, and genetic patterns if we are to elucidate the key processes underlying \( Bd \) disease dynamics and evolution of resistance in free-living amphibians. Identifying immunogenetic correlates of chytridiomycosis outcomes provides a mechanism to explain variable host susceptibility among individuals, populations, and perhaps species. Analyses of \( Bd \) dynamics within and across amphibian species, pathogen strains, and geographic regions, which have
been important in elucidating environmental and ecological predictors of disease dynamics (Briggs et al. 2010; Rohr et al. 2011), may also benefit from incorporation of host genetic contributions. Immunogenetic markers for Bd resistance also provide a potential tool for proactive conservation via marker-assisted selection (Woodhams et al. 2011), a potentially powerful tool in mitigating the impact of this global pandemic on amphibian biodiversity. Finally, identifying immunogenetic hallmarks of Bd resistance in natural populations is a critical step towards species recovery, as the global spread and persistence of Bd means that wild populations must ultimately evolve disease resistance to achieve long-term species survival.

Methods

Field Surveys. We surveyed eight L. yavapaiensis populations for Bd and chytridiomycosis during winter months (January–February) of 2007-2011 (Savage et al. 2011a) and pooled samples across the five survey years. Using a standardized protocol (Hyatt et al. 2007) we swabbed the epidermis of live, sick and dead L. yavapaiensis individuals with sterile fine-tip swabs (Medical Wire & Equipment Co. MW113). We categorized each dead individual that tested positive for Bd infection as a chytridiomycosis mortality event. Additionally, we collected any individual with signs of chytridiomycosis (i.e., skin redness, lethargy, failure to seek shelter, and loss of righting ability) for overnight observation, and also categorized these as chytridiomycosis-induced mortality events if death occurred within 24h and the individual tested positive for Bd. We estimated Bd and chytridiomycosis mortality prevalence and 95% Clopper-Pearson binomial confidence intervals (Clopper & Pearson 1934) for each L. yavapaiensis population sample.

Microsatellite genotyping. We genotyped 282 L. yavapaiensis individuals at 14
previously characterized microsatellite loci (Savage & Jaeger 2008). Template DNA for microsatellite genotyping was extracted using a 5% Chelex 100 solution (Bio-Rad Laboratories) with 0.5µg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. The supernatant from these extractions was used directly as template for PCR amplification, performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different labels or non-overlapping size ranges were multiplexed and run on a 3730 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined by comparison with a LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems). We used GENEPOP version 3.4 (Raymond & Rousset 1995) to calculate observed and expected heterozygosities and test for deviations from Hardy-Weinberg equilibrium (HWE) at each locus and population locality using a Monte Carlo chain method (1000 dememorizations, 100 batches, 1000 iterations; Guo & Thompson 1992) and a Bonferroni correction for multiple tests (adjusted $P = 0.00022$).

**MHC amplification, cloning and sequencing.** We extracted genomic DNA from toe clips (preserved in ethanol) from 128 *L. yavapaiensis* individuals using Qiagen DNeasy kits (Qiagen, Valencia, CA). The majority (108) of the individuals were sampled in winter, but for populations with prohibitively small winter sample sizes (CIC, TV and WC), we sampled 20 additional frogs collected in summers of 2006 and 2007. We used a degenerate frog class II MHC β1 forward primer (MHC-F; Hauswaldt et al. 2009) and a ranid frog MHC β1 intron-specific reverse primer (B1intron2_R; Kiemnec-Tyburczy et al. 2010) to amplify 249 base pairs (bp) of exon 2 and 189 bp of adjacent 3' flanking intron. Amplifications were performed on genomic DNA for 35 cycles (95°C for 50 s; 60°C for 45
s; and 72°C for 1 min) with standard reaction conditions. We cloned the PCR products from all reactions into pGEM® T vectors (Promega, Madison, WI) and transformed recombinant DNA into TOP-10 E. coli cells (Invitrogen). Cells were grown on Luria agar plates for 18 hours at 37°C. We used blue/white screening to randomly choose 6-24 positive clones from each transformation and amplified them using M13 primers for 30 cycles (95°C for 1 min; 55°C for 30 s; and 72°C for 30 s) with standard reaction conditions. PCR products were visualized on a 1% agarose gel and products in the appropriate size range were purified using an alkaline phosphatase-exonuclease reaction. The products were sequenced on an automated 3730 DNA analyzer using Big Dye v3.1 chemistry (Applied Biosystems). We screened each MHC sequence and only included those obtained from at least two clones. For each individual, sequences recovered only once with ≤ 2 nucleotide differences to other cloned sequences were attributed to PCR/cloning errors and discarded. After excluding these sequences, no more than two unique MHC alleles were recovered from any individual. We used translated amino acid queries in GenBank (tBLASTx) to confirm MHC class II homology and then aligned the sequences using Sequencher v. 4.10 (Gene Codes Corporation, Ann Arbor, MI).

**Genealogy reconstruction.** We first tested the alignment of MHC sequences for evidence of recombination using the Single Breakpoint (SBP) method (Pond et al. 2006). We found no evidence of recombination, thus we performed a Bayesian analysis to reconstruct genealogical relationships among MHC alleles. We used MHC class II β1 exon 2 sequences from the frogs *Silurana laevis* and *S. tropicalis* (GenBank accession numbers NM_001114771 and NM_001045794) as outgroups. Model parameters were determined using the Akaike information criteria (AIC) in jModeltest (Posada 2008). We used the best-fit model (GTR+I+G) to estimate a 95% credible set of rooted MHC
genealogies in the software MrBayes 3.1 (Ronquist & Huelsenbeck 2003). We ran two separate analyses in MrBayes for $1 \times 10^7$ generations and sampled every 500th generation of the Markov chain. We used Tracer v1.4 to assess the stationarity of model parameters, convergence of model parameters between runs, the number of burn-in samples, and the effective sample sizes for each parameter.

**Tests of selection.** We ran tests of selection using the HyPhy software package (Kosakovsky Pond *et al.* 2005). We used the Bayesian genealogy as our input tree, excluding the outgroup sequences. We first used the PARRIS method to test for positive selection in the entire alignment (Scheffler *et al.* 2006). This method expands on other maximum likelihood methods for detecting positive selection by allowing substitution rates to vary across sites, while accounting for recombination by detecting breakpoints when measuring nonsynonymous and synonymous substitution rates. Second, we used the Evolutionary Fingerprinting method to infer the number of positive selection rate classes and the intensity of selection in each rate class (Kosakovsky Pond *et al.* 2010). Third, we tested for residue-specific positive selection across evolutionary lineages of MHC class IIB exon 2 sequences using the most conservative maximum likelihood method, Single Likelihood Ancestral Counting (SLAC). This method infers ancestral codon states and calculates normalized expected and observed nonsynonymous (dN) and synonymous (dS) substitutions at each site (Kosakovsky Pond & Frost 2005).

**Selection and genetic differentiation among populations**

We estimated $F_{ST}$ across all population pairs for (i) 14-locus microsatellite genotypes, (ii) PBR exon genotypes, and (ii) PBR intron genotypes using Arlequin version 3.5 (Excoffier & Lischer 2010). For each population, we calculated observed and expected heterozygosity, nucleotide diversity ($\pi$), theta($\theta$), and performed Ewens-Watterson tests.
separately for PBR exon, intron and microsatellite genotypes using Arlequin version 3.5 (Excoffier & Lischer 2010). We also performed Tajima’s $D$ and Fu’s $F_S$ tests on PBR exon and intron genotypes.

**Statistical Analyses**

Differences in *Bd* infection intensity and nucleotide diversity across populations were assessed using Student’s t-tests implemented in JMP software, version 9.0 (SAS). We calculated Relative Risk (RR; Sistrom & Garvan 2004) using equation (1):

\[
RR = \left( p^+ \times c^- \right) + \left( p^- \times c^+ \right),
\]

where $p^+$ is the frequency of frogs with MHC allele $x$ that died from chytridiomycosis, $p^-$ is the frequency of frogs with MHC allele $x$ that did not die from chytridiomycosis, $c^-$ is the frequency of frogs without MHC allele $x$ that died from chytridiomycosis, and $c^+$ is the frequency of frogs without MHC allele $x$ that did not die from chytridiomycosis. Significance of each RR value was assessed using Fisher’s exact test, with sequential Bonferroni correction for multiple comparisons (Rice 1989).

**Acknowledgements:** We thank J.Q. Richmond, K. Kiemnec-Tyburczy, and M. Lenker for help in the laboratory; D. Caldwell, M. Sredl, M. Haberstitch, R. Rogers and M. Lawrence for field support; and members of the Zamudio lab for discussions and comments. This research was supported by an NSF Doctoral Dissertation Improvement Grant (DEB-0909013) to A.E.S. and a Population and Evolutionary Process NSF Grant (DEB-0815315) to K.R.Z.
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Kosakovs


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Figure S1.1: Map of *Lithobates yavapaiensis* population localities in Arizona, USA that were sampled in summers and winters of 2006 – 2010.
Figure S3.1: (A) Egg mass collection localities in Arizona, USA, for the five Lithobates yavapaiensis populations used in experimental Bd-infections. Site abbreviations follow Figure 1. (B) Photo of Aravaipa Canyon, showing typical L. yavapaiensis habitat. (C) Image of a Bd-infected frog found dead with signs of chytridiomycosis at Cienega Creek. (D) Image of a healthy frog from Cienega Creek.
Figure S3.2: Number of microsatellite alleles recovered for each *Bd*-infected *Lithobates yavapaiensis* clutch. Colors represent each of the 14 genotyped microsatellite loci. The dotted line shows the threshold above which multiple paternity was inferred.
**Figure S3.3:** Alignment of 33 MHC class IIB alleles recovered from 99 *Bd*-infected *Lithobates yavapaiensis* individuals. Alleles were aligned to *Silurana (Xenopus) laevis* and *Lithobates (Rana) temporaria* partial MHC class IIB sequences from GenBank (Accession numbers EF210752.1 and FJ876299.1, respectively).
Table S1. Summary of *Lithobates yavapaiensis* sampling localities, previously documented die-offs, and for field surveys spanning July 2006 through July 2010, seasonal sample size, seasonal *Bd* infection, and winter chytridiomycosis mortality. The number of recaptures are shown in parentheses.

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<th>Locality</th>
<th>Abbr.</th>
<th>UTM (Zone 12; NAD27)</th>
<th>Elevation (m)</th>
<th>Documented die-offs</th>
<th>N_SUMMER</th>
<th>N_WINTER</th>
<th>N_TOTAL</th>
<th>Summer <em>Bd</em> infect. (95%CI)</th>
<th>Winter <em>Bd</em> infect. (95%CI)</th>
<th>Winter <em>Bd</em> mort. (95%CI)</th>
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*Nature Conservancy property with long-term monitoring (LTM) beginning in the indicated year

*Bradley et al. 2002

*Rosen and Schwalbe 2002*
**Table S2.2:** GENEPOP tests of deviations from Hardy-Weinberg Equilibrium for each locus and population. *P*-values are indicated for all significant deviations (adjusted \( P=0.00022 \)).

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Table S2.3: (A) Recent migration among populations estimated using BAYESASS, populations 1-7.

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Table S2.3: (B) Recent migration among populations estimated using BAYESASS, populations 8-13.

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<td>0.001 (0.005)</td>
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<td>0.001 (0.011)</td>
<td>0.980 (0.943-1)</td>
<td>0.001 (0.001)</td>
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<td>0.002 (0.009)</td>
<td>0.001 (0.007)</td>
<td>0.987 (0.967-1)</td>
<td>0.001 (0.006)</td>
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<td>0.001 (0.006)</td>
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Table S2.4: Model selection for genetic and environmental factors influencing mortality prevalence, *Bd* infection prevalence, and *Bd* infection intensity of *L. yavapaiensis* in Arizona. We compared all possible models using Akaike Information Criterion (AICc); Interactions among variables were disregarded; five best models are reported for each dataset.

### Bd Mortality Prevalence

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### Bd Infection Prevalence

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### Bd Infection Intensity

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Best predictors are as follows: for neutral loci, observed heterozygosity (H₀) and number of private alleles (Nº PRIVATE ALLELES); for the outlier locus, observed heterozygosity (C110- H₀); for environmental variables: mean diurnal temperature range [mean of monthly max temp - min temp] (BIO2), minimum temperature of coldest month (BIO6), temperature annual range [max temperature of warmest month – min temperature of coldest month] (BIO7), mean temperature of wettest quarter (BIO8), mean temperature of coldest quarter (BIO11), annual precipitation (BIO12), precipitation of wettest month (BIO13), precipitation seasonality [coefficient of variation] (BIO15), precipitation of wettest quarter (BIO16), and precipitation of driest quarter (BIO17).
Table S3.1: List of lab-reared, *Bd*-infected *Lithobates yavapaiensis* individuals, housing conditions, change in mass over the course of the experiment, maximum *Bd* infection intensity (in genome equivalents), and MHC class IIB peptide-binding region genotypes. Surviving individuals are shown in bold.

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<th>Replicate</th>
<th>Clutch</th>
<th>Days survived</th>
<th>Survived?</th>
<th>No./cage</th>
<th>0 DPI mass (g)</th>
<th>death/25 DPI mass (g)</th>
<th>change in mass</th>
<th>PBR het or hom</th>
<th>Max. Inf. Intensity</th>
<th>PBR Allele 1</th>
<th>PBR Allele 2</th>
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Table S3.2: Terminal branch evolution among 33 PBR sequences from five *L. yavapaiensis* populations experimentally infected with *Bd*. A Maximum Likelihood (ML) phylogeny of the 82-codon PBR alignment was used as the input tree for selection on particular codons within branches of the phylogeny. We used three different codon-based methods, all implemented with the HyPhy statistical software (66). The most conservative method, SLAC, infers ancestral codon state and then calculates normalized expected and observed nonsynonymous (dN) and synonymous (dS) substitutions at each site (66). The FEL method directly estimates rates of nonsynonymous and synonymous substitutions at each site, while the REL approach fits a distribution of substitution rates across sites and then infers the rate at which each site evolves (66). When the normalized difference between non-synonymous and synonymous substitutions (dN-dS) equals 0, the site is neutrally evolving; when dN-dS >0, the site is positively selected; and when dN-dS <0, the site is negatively selected.

**Single Likelihood Ancestral Counting (SLAC)**

<table>
<thead>
<tr>
<th>Terminal branch of ML phylogeny</th>
<th>Codon site under positive selection</th>
<th>Normalized dN-dS</th>
<th>P-value</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leading to allele EE</td>
<td>3</td>
<td>18.1</td>
<td>0.09</td>
<td>Gly → His</td>
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**Fixed-Effects Likelihood (FEL)**

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<th>Codon site under positive selection</th>
<th>Normalized dN-dS</th>
<th>P-value</th>
<th>Amino acid change</th>
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</thead>
<tbody>
<tr>
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<td>3</td>
<td>1.1</td>
<td>0.03</td>
<td>Gly → His</td>
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<tr>
<td>Leading to allele EE</td>
<td>18</td>
<td>0.93</td>
<td>0.03</td>
<td>Ser → Lys</td>
</tr>
<tr>
<td>Leading to allele Q</td>
<td>46</td>
<td>0.74</td>
<td>0.04</td>
<td>Leu → Val</td>
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**Random-Effects Likelihood (REL)**

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<th>Codon site under positive selection</th>
<th>Normalized dN-dS</th>
<th>Posterior Probability</th>
<th>Amino acid change</th>
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<tr>
<td>Leading to allele EE</td>
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<td>3.21</td>
<td>0.99</td>
<td>Gly → His</td>
</tr>
<tr>
<td>Leading to allele EE</td>
<td>18</td>
<td>3.32</td>
<td>0.99</td>
<td>Ser → Lys</td>
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Table S3.3: Survival from hatching through start of experimental infections for lab-reared *Lithobates yavapaiensis*.

<table>
<thead>
<tr>
<th>Clutch</th>
<th>Number of larvae hatched (%)</th>
<th>Number of larvae reaching Gosner stage 45 (%)</th>
<th>Number of frogs surviving metamorphosis (post-Gosner stage 46; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIC1</td>
<td>143 (100%)</td>
<td>118 (83%)</td>
<td>32 (27%)</td>
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<tr>
<td>CIC2</td>
<td>134 (100%)</td>
<td>123 (92%)</td>
<td>24 (20%)</td>
</tr>
<tr>
<td>AC1</td>
<td>188 (100%)</td>
<td>91 (48%)</td>
<td>14 (15%)</td>
</tr>
<tr>
<td>AC2</td>
<td>205 (100%)</td>
<td>119 (58%)</td>
<td>14 (12%)</td>
</tr>
<tr>
<td>MR1</td>
<td>195 (100%)</td>
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<td>5 (57%)</td>
</tr>
<tr>
<td>MR2</td>
<td>118 (100%)</td>
<td>2 (2%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>MR3</td>
<td>0 (0%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>WC1</td>
<td>96 (20%)</td>
<td>54 (56%)</td>
<td>7 (13%)</td>
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<tr>
<td>WC2</td>
<td>255 (100%)</td>
<td>167 (65%)</td>
<td>16 (10%)</td>
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<tr>
<td>WC3</td>
<td>117 (100%)</td>
<td>41 (35%)</td>
<td>5 (12%)</td>
</tr>
<tr>
<td>SM1</td>
<td>247 (100%)</td>
<td>159 (64%)</td>
<td>17 (11%)</td>
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<tr>
<td>SM2</td>
<td>155 (100%)</td>
<td>98 (63%)</td>
<td>13 (13%)</td>
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<td>SM3</td>
<td>253 (100%)</td>
<td>115 (45%)</td>
<td>9 (8%)</td>
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<tr>
<td>Total</td>
<td>1810</td>
<td>1044 (58%)</td>
<td>158 (15%)</td>
</tr>
<tr>
<td>Mean</td>
<td>144 (85%)</td>
<td>87.5 (55%)</td>
<td>13.2 (16%)</td>
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**Table S4.1:** Pairwise estimates of $F_{ST}$ (upper) and $D$ (lower) among *Lithobates yavapaiensis* study populations. All values are significant: for pairwise $F_{ST}$ values, adjusted $P = 0.000549$; for pairwise $D$, 95% confidence intervals did not include zero.

<table>
<thead>
<tr>
<th></th>
<th>AC</th>
<th>TV</th>
<th>CIC</th>
<th>MR</th>
<th>SM</th>
<th>WC</th>
<th>HR</th>
<th>SS</th>
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<tbody>
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<td>–</td>
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<td>0.4054</td>
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<tr>
<td>CIC</td>
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<td>0.3373</td>
<td>–</td>
<td>0.1781</td>
<td>0.3116</td>
<td>0.3559</td>
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<td>0.2531</td>
<td>0.2561</td>
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<tr>
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<td>0.8592</td>
<td>0.7644</td>
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<td>–</td>
<td>0.2039</td>
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<td>0.4144</td>
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<td>0.1935</td>
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<td>0.7840</td>
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<td>0.6099</td>
<td>0.6239</td>
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**Table S4.2:** Pairwise Euclidean distances among *Lithobates yavapaiensis* study populations in kilometers.

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<th>CIC</th>
<th>MR</th>
<th>SS</th>
<th>SM</th>
<th>WC</th>
<th>HR</th>
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<tbody>
<tr>
<td>AC</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TV</td>
<td>73</td>
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<td></td>
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<td></td>
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<tr>
<td>CIC</td>
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<tr>
<td>MR</td>
<td>65</td>
<td>40.5</td>
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<tr>
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<td>290</td>
<td>66</td>
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<td></td>
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<tr>
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<td>220</td>
<td>245</td>
<td>233</td>
<td>132</td>
<td>202</td>
<td>79</td>
<td>–</td>
</tr>
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</table>
APPENDIX 3:

Isolation and characterization of microsatellite markers in the lowland leopard frog
(Rana yavapaiensis) and the relict leopard frog (R. onca), two declining frogs of the
North American desert southwest

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14853, USA
† School of Life Sciences and Public Lands Institute, University of Nevada, 4505
Maryland Parkway, Las Vegas, NV 89154-4004

Abstract

We characterized 15 microsatellite loci for the lowland leopard frog (Rana
yavapaiensis) and the relict leopard frog (R. onca) for future studies of population
genetic structure and relatedness. Analysis of 20 individuals from single populations
of each species showed that all markers were polymorphic in at least one species.
Observed and expected heterozygosities ranged from 0 to 0.94 and 0.11 to 0.85,
respectively, and there were 3 to 11 alleles per locus. No loci were in linkage
disequilibrium, but six loci deviated significantly from Hardy-Weinberg equilibrium,
and the presence of a null allele was detected in two of these loci.

Manuscript

The lowland leopard frog (Rana yavapaiensis) and the relict leopard frog (R. onca) are sister species within the R. pипiens complex (Pantherana) (Hillis and Wilcox
Rana onca was once considered extinct (Jennings 1988), but recently, several extant populations were confirmed as R. onca based on mitochondrial haplotypes, assessment of randomly amplified polymorphic DNA (RAPD markers), and morphology (Jaeger et al. 2001). Taxonomic designations may be uncertain, however, because these two species are almost identical at mitochondrial DNA markers (approximately 98.8% similarity at the 12 S and 16 S genes), a level of divergence comparable to that among other ranid subspecies (Hillis and Wilcox 2005).

Both R. yavapaiensis and R. onca are species of conservation concern. Rana onca was known from only 24 locations in Utah, Nevada and Arizona prior to the 1970s, but by 2001 its range dwindled to 5 localities in Nevada (Bradford et al. 2004). In contrast, R. yavapaiensis is still patchily distributed throughout much of Arizona and Sonora (Hillis 1988), although the species has also experienced considerable declines and extirpations since the 1970s (Sredl et al. 1997), at least some of which were caused by the fungal disease chytridiomycosis (Bradley et al. 2002). Characterization of fine-scale levels of genetic variability within and among these species is therefore critical to establish conservation priorities and to inform questions of species designation.

To accomplish this objective, 15 microsatellite loci were isolated and characterized from two enriched partial genomic libraries (Hamilton et al. 1999), one prepared with tissue from an adult R. yavapaiensis individual from Maricopa County, Arizona, and one prepared with tissue from an adult R. onca individual from Clark County, Nevada. For the R. yavapaiensis library, genomic DNA was extracted using a Qiagen DNeasy tissue kit, followed by digestion with AluI and HaellII (New England Biolabs). The linked genomic fragments were enriched for microsatellites with
biotinylated dimer, trimer and tetramer probes bound to streptavidin-coated magnetic beads (Dynabeads, Dynal Biotech). Subsequently, the microsatellite-containing DNA fragments were magnetically captured. These fragments were amplified by polymerase chain reaction (PCR) using linker-specific primers, followed by digested with *NheI* (New England Biolabs). Resulting fragments were cloned into pUC19 vector and transformed with DH5α competent cells (Invitrogen). Colonies were grown on X-Gal/IPTG-coated agar and transferred to Magna Lift nylon membranes (Osmonics Inc.) that were probed with the same di-, tri- and tetramer radiolabeled repeats. Positive clones were transferred to 30 µl of de-ionized water, boiled at 99°C for 5 min, and 1 µl of the resulting solution was used as template for PCR amplification with vector-specific primers (M13 F and R). Amplification products >300 base pairs were sequenced in one direction with the M13 F primer using dGTP BigDye terminator cycle sequencing components on a 3100 Genetic Analyzer (Applied Biosystems). For the *R. onca* library, a private company (Genetic Identification Services) was employed to isolate, clone and sequence microsatellite-containing DNA fragments.

PCR primers were designed in the flanking regions of 22 microsatellites from the *R. yavapaiensis* library using SEQMAN version 5.05 (DNAStar Inc.) and 46 microsatellites from the *R. onca* library using the program DESIGNERPCR version 1.03 (Research Genetics, Inc.). Eight *R. yavapaiensis* from 4 sites and 8 *R. onca* from 4 sites, representing a broad area of the current range for each species, were screened for amplification reliability and microsatellite polymorphism. Of the evaluated primers, 15 polymorphic loci were identified. These loci were used for a broader assessment of population and species differentiation.

Genotyping was conducted for 20 *R. yavapaiensis* individuals from a single
location on the Santa Maria River (Yavapai County, Arizona) and 20 R. onca individuals from Bighorn Sheep Spring along the Colorado River (Clark County, Nevada). DNA was extracted from toe clips in 150μl of a 5% Chelex-100 solution (Bio-Rad) with 1μl Proteinase K by incubation at 55 °C for 180 min and 99 °C for 10 min; supernatants were used directly as PCR templates. Each PCR had a total volume of 10μl, consisting of 1μl of each template, 0.1μl Taq DNA polymerase (5U/μl) (Roche), 1.5μl 10X PCR buffer (100mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl), 0.25μl dNTPs (40mM), and 0.5μl of each primer (10μM). Forward primers were 5’-labeled with a fluorescent dye (Applied Biosystems). Amplification was performed in a Hybaid PCR thermal cycler under the following conditions: 5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at primer specific annealing temperatures (Table 1), 1 min extension at 72 °C; and a final extension of 75 °C for 5 min. Amplified products with different labels or nonoverlapping size ranges were multiplexed and electrophoresed on a 5% polyacrylamide gel on a 3100 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined with the LIZ-500 standard using GENEMAPPER version 3.5 (Applied Biosystems). Tests of significant deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), and calculations of the number of alleles (A), observed (H₀) and expected (Hₑ) heterozygosities were performed using GENEPOP version 3.4 (Raymond and Rousset 1995).

The majority of the microsatellite loci identified were tetramers or complex repeats mostly containing tetramers, while only two loci were pure dimers (Table 1). All 15 loci were polymorphic among R. yavapaiensis individuals, while only 10 loci were polymorphic among the R. onca sampled. Lower genetic diversity was expected in R. onca because the current remnant populations comprise a very small
geographic range (Bradford et al. 2004). The total number of alleles per locus across both species ranged from 3 to 11 (mean 6.7), with 2 to 9 alleles per locus in *R. yavapaiensis* (mean 5.1) and 1 to 5 alleles per locus in *R. onca* (mean 2.6). For 9 of the loci, 1 to 3 alleles were shared among *R. yavapaiensis* and *R. onca* individuals. Across all samples, observed and expected heterozygosities ranged from 0 to 0.94 and 0.11 to 0.85, respectively.

No significant LD was detected among any pair of loci; however, six loci showed significant deviations from HWE. Loci *RoC4*, *RoD125*, and *RoD122* showed significant heterozygote deficiency for *R. yavapaiensis* only, loci *RoD120* and *RoD124* showed significant heterozygote deficiency in population samples from both species, and locus *RyTET-e* showed significant heterozygote excess for *R. onca* only. The program MICRO-CHECKER (van Oosterhout et al. 2004) was used to further evaluate these loci, and the presence of a null allele was detected at loci D120 and D122 for the *R. yavapaiensis* population sample. The 15 loci characterized here will be used in range-wide studies of population structure within and among *R. yavapaiensis* and *R. onca* populations, providing a comprehensive understanding of relatedness and conservation priorities for these threatened species.

**Acknowledgements:** This project was funded by a NSF Graduate Research Fellowship and a National Geographic Society Young Explorers Grant to A.E.S. and the Clark County Desert Conservation Program funded by Southern Nevada Public Lands Management Act (project # 2005-UNLV-575-P) to J.R.J. We thank S. Bogdanowicz and the Evolutionary Genetics Core Facility for help with microsatellite development, M.C. Duryea for help with data analysis, and David Bradford and Brett Riddle for their efforts in support of this project.
REFERENCES


Arizona Game and Fish Department, Phoenix, AZ.


*Molecular Ecology Notes, 4*, 535-538.
<table>
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<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Primer sequence (5’ - 3’)</th>
<th>Tm (°C)</th>
<th>Size range</th>
<th>N</th>
<th>A&lt;sub&gt;Ne&lt;/sub&gt;</th>
<th>A&lt;sub&gt;Ne&lt;/sub&gt;</th>
<th>P&lt;sub&gt;0&lt;/sub&gt;</th>
<th>A&lt;sub&gt;Ne&lt;/sub&gt;</th>
<th>A&lt;sub&gt;Ne&lt;/sub&gt;</th>
<th>P&lt;sub&gt;0&lt;/sub&gt;</th>
<th>p</th>
<th>R&lt;sub&gt;p&lt;/sub&gt; value</th>
<th>R&lt;sub&gt;p&lt;/sub&gt; value</th>
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<td>(GT&lt;sub&gt;26&lt;/sub&gt;)(AC&lt;sub&gt;12&lt;/sub&gt;)(GT&lt;sub&gt;12&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>E. (6-FAM)TACAGACGGTACCTCTCTCTCTC</td>
<td>55</td>
<td>192-243</td>
<td>37</td>
<td>5</td>
<td>3</td>
<td>7</td>
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<td>0.84/0.83</td>
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<td>59</td>
<td>184-239</td>
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<td>8</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>0.80/0.82</td>
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<td>0.12/0.01</td>
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<td>E. (PET)TACAGACGGTACCTCTCTCTCTC</td>
<td>60</td>
<td>124-156</td>
<td>37</td>
<td>8</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>0.80/0.82</td>
<td>0.38</td>
<td>0.25/0.20</td>
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<td>2</td>
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<td>0.25/0.20</td>
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<td>60</td>
<td>102-124</td>
<td>38</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>2</td>
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<td>60</td>
<td>192-251</td>
<td>38</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>0.75/0.33</td>
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<td>60</td>
<td>192-251</td>
<td>38</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>0.75/0.33</td>
<td>0.50</td>
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<td>E. (6-FAM)TACAGACGGTACCTCTCTCTCTC</td>
<td>65</td>
<td>212-289</td>
<td>40</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0.53/0.47</td>
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<td>65</td>
<td>212-289</td>
<td>40</td>
<td>7</td>
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<td>8</td>
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<td>55</td>
<td>124-224</td>
<td>39</td>
<td>2</td>
<td>5</td>
<td>7</td>
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<td>0.41/0.25</td>
<td>0.11</td>
<td>0.51/0.43</td>
<td>0.05</td>
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<td>E. (PET)TACAGACGGTACCTCTCTCTCTC</td>
<td>61</td>
<td>212-289</td>
<td>39</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>0.41/0.25</td>
<td>0.11</td>
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<td>55</td>
<td>124-224</td>
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<td>7</td>
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<td>0.41/0.25</td>
<td>0.11</td>
<td>0.51/0.43</td>
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<td>212-289</td>
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<td>0.41/0.25</td>
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<td>61</td>
<td>212-289</td>
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<td>50</td>
<td>182-244</td>
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<td>166-186</td>
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<td>0.48/0.55</td>
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Note: All values represent significance values for tests of deviation from Hardy-Weinberg equilibrium. Significant deviations are indicated in bold. NA: Not applicable.